SOME ASPECTS OF PYRUVATE METABOLISM IN MAMMALIAN LIVER

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CHAPTER I

INTRODUCTION

The unique ability of the liver to reversibly convert glucose into a readily-available storage form has been recognized for over a century (1,2). It has only been in the last decade, however, that the full enzymatic basis for the liver's control of blood glucose levels via glycogenolysis, gluconeogenesis, and glycolysis has become clear (3). The importance of the liver's regulatory role in maintaining the physiological well-being of the animal becomes apparent when one realizes that the entire nervous system as well as the cells of the blood utilize glucose as their main source of energy.

Since a major function of the liver is to provide glucose for the organs of the body other than itself, it is reasonable to expect that the liver would not readily use glucose as a source of energy. Krebs states that the high gluconeogenic capacity of the liver is in marked contrast to its low glycolytic capacity (3). The contribution of glucose to total hepatic CO_2 production both *in vivo* and *in vitro* is very small, indicating the quantitatively minor role of glucose in hepatic oxidation (10,11,12,13,14). Burch (15) has observed that the changes in levels of the various enzymes concerned with carbohydrate metabolism during hepatic development and differentiation are indicative of a decrease in capacity for glucose utilization and glycolysis, and an

increase in capacity for gluconeogenesis and glycogenolysis. According to Fritz (16) and Pesch and Topper (11), the primary energy source in hepatic metabolism is the oxidative breakdown of fatty acids, lactate, and other gluconeogenic precursors, via the tricarboxylic acid cycle.

The liver is also differentiated from other metabolic systems of the body since it is the sole major site of ketone body production in higher animals, and it is unable to oxidize acetoacetate (4). It has been postulated that the ketone bodies are transport forms of readily available energy which is distributed by the liver to other tissues via the blood stream (4,6). The liver raises plasma ketone body and free fatty acid levels when glucose is not available in sufficient quantity in such conditions as starvation or low-carbohydrate diets (4). This ability of the liver to provide readily oxidizable substrates for the intact organism under varying nutritional conditions is a part of the liver's role in the control of normal whole-body metabolism.

The rate of gluconeogenesis, i.e., synthesis of carbohydrate from amino acids or from other precursors such as lactate, pyruvate, succinate, or propionate, is quite variable and is regulated according to the needs of the organism. The fact that the rate of gluconeogenesis adapts to the physiological needs of the body implies that the body must possess rate controlling mechanisms. These control mechanisms are of two kinds: one regulates the amount of the key enzymes of gluconeogenesis, and one regulates the activities of the existing enzymes (3,6). A considerable amount of literature is concerned with the important role of glucocorticoid hormones and nutritional conditions

in controlling gluconeogenesis by regulating either the amounts of enzyme or their activities, and no attempt will be made to survey this material (8,9), the exception being those references which are pertinent to the metabolism of pyruvate.

In 1937 Krebs and Johnson (17) first proposed the tricarboxylic acid cycle as a scheme for the oxidative metabolism of carbohydrate. Since that time it has been found that the tricarboxylic acid cycle is the common exergonic pathway through which fatty acids and amino acids as well as carbohydrates are oxidized to CO_2 and H_2O (18). These relationships are illustrated in Figure 1. (3).

As shown in Figure 1, pyruvate can be metabolized via several different pathways. Which route assumes metabolic importance is determined by enzyme concentrations and activities which, are, in turn, influenced by dietary conditions as well as substrate and cofactor concentrations.

It has become a widely accepted concept that gluconeogenesis is not merely a reversal of glycolysis, but in fact, involves a number of additional energy requiring steps to circumvent difficulty reversible reactions (19). Krebs (19) has argued on energetic grounds that the conversion of P-enolpyruvate[1] to pyruvate via pyruvate kinase (EC 2.7.1.40) is not a reversible reaction. Additional data which supports this concept are the low activity of the enzyme for the conversion of pyruvate to P-enolpyruvate, and the high apparent Km value for pyruvate (Km = 20 mM), which is considerably above probable physiolog-

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[1] Abbreviations are in accordance with the IUPAC-IUB Combined Commission on Biochemical Nomenclature, J. Biol. Chem., 241, 527 (1966).

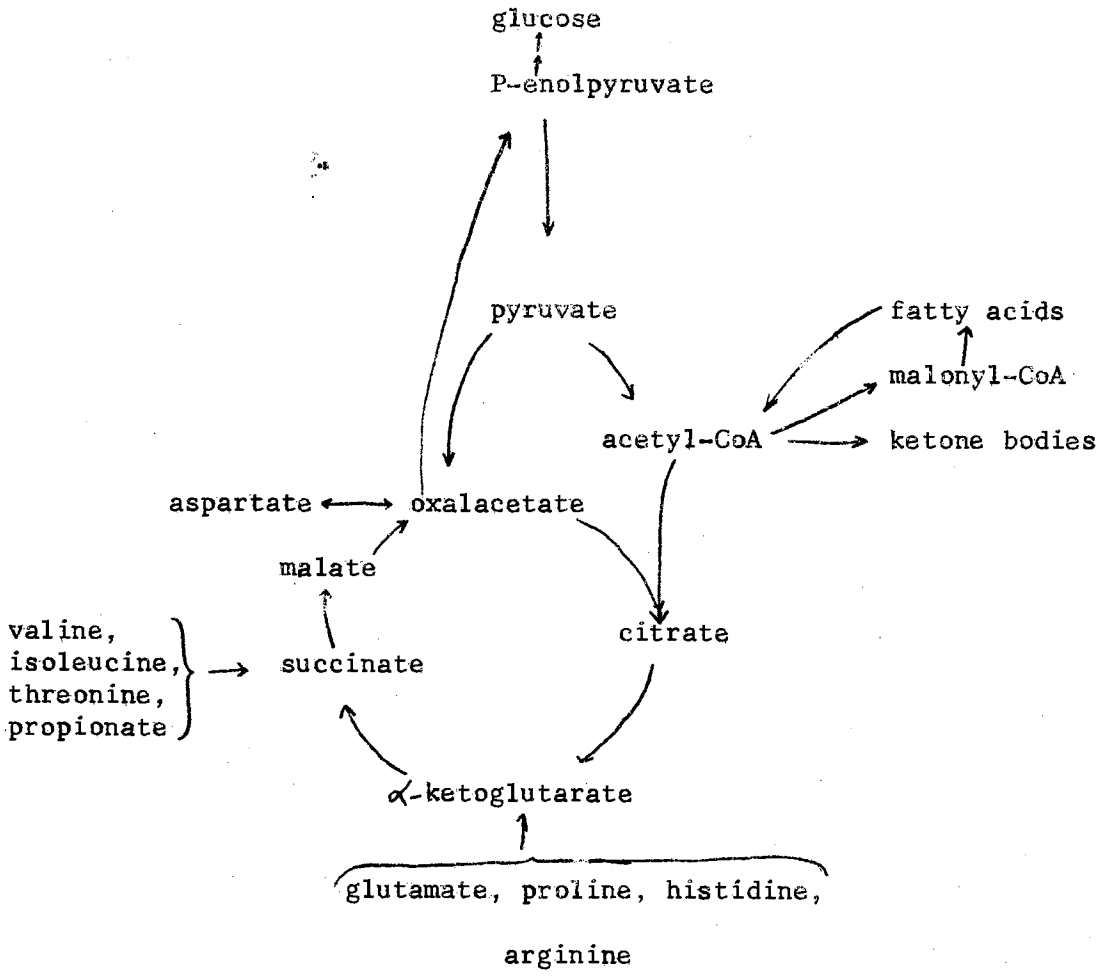

Figure 1. Illustration of the Common Exergonic Pathways of Amino Acids, Fatty Acids, and Carbohydrates.

ical levels of pyruvate (20). The concentration of pyruvate in the cell is approximately 0.02 mM. Labeling patterns found in hepatic glucose when labeled pyruvate is given as substrate also substantiate the nonreversibility of the reaction catalyzed by liver pyruvate kinase (21,23,23,24,25). Hiatt et al. (22) have shown that in muscle slices pyruvate-2-^{14}C is incorporated with limited randomization into glucose. From these data they proposed that in muscle there is a direct conversion of pyruvate to P-enolpyruvate. Lack of equilibration of the dicarboxylic acids by fumarase (EC 4.2.1.2) could also account for the lack of randomization of labeling in muscle glycogen. The inability of Keech and Utter (26) to demonstrate any pyruvate carboxylase (EC 6.4.1.1) in rabbit skeletal muscle could be regarded as evidence against the formation of a four-carbon intermediate from pyruvate. However, the observed labeling patterns in muscle glutamate after the administration of pyruvate-2-^{14}C (27,28), alanine-2-^{14}C (29,30), and serine-2-^{14}C (31) indicate that a four-carbon unit is an intermediate formed in the metabolism of these compounds in muscle tissue in vivo. The amount of labeling in carbon 4 of brain glutamate represents about 18 percent of the total labeling in the compound (32). Muscle glutamate, however, has only 1 to 2 percent of its total labeling in carbon 4. This large amount of randomization in carbon 4 of brain glutamate relative to carbon 5 (60-70%) suggests that pyruvate-2-^{14}C is converted by the liver to blood glucose-1,2,5,6-^{14}C prior to being metabolized by the brain (32). The low amount of randomization in carbon 4 of muscle glutamate relative to that found in carbon 5 suggests that blood glucose-1,2,5,6-^{14}C does not contribute greatly to the labeling

of muscle glutamate, and that muscle is capable of conserving carbohydrate for dicarboxylic acid synthesis, possibly via the malic enzyme (EC 1.1.1.40).

The sequence of reactions by which P-enolpyruvate is formed from pyruvate in the liver involves several different enzymes and cofactors (34). Some of these reactions occur in different compartments of the cell (34). For example, pyruvate is carboxylated to form oxalacetate (35) in the mitochondria (36,37,38), but the fact that P-enolpyruvate carboxykinase (EC 4.1.1.32) is found mainly in the cytoplasm in most mammalian species necessitates the transport of oxalacetate to that cellular compartment before P-enolpyruvate can be formed (39,40). Oxalacetate, however, is not transported across the mitochondrial membrane to any great extent (34). Present evidence indicates that oxalacetate is partially reduced to malate and partially transaminated to form aspartate (41,42), and that these two compounds then freely diffuse from the mitochondria into the cytoplasm where they are reconverted to oxalacetate (41). The synthesis of P-enolpyruvate from pyruvate via malate and the liver malic enzyme accounts for only a small fraction of the four-carbon acids required for gluconeogenesis (43).

Citrate could conceivably be a potential source of four-carbon units for gluconeogenesis (44), but the finding of Kornacker and Lowenstein (45) that the activity of the citrate cleavage enzyme is decreased during fasting, coupled with the finding that in soluble extracts of rat liver citrate, in comparison to malate, is a poor precursor of P-enolpyruvate (46), serve to rule out the function of citrate as a source of four-carbon units for gluconeogenesis. Figure 2 gives the scheme

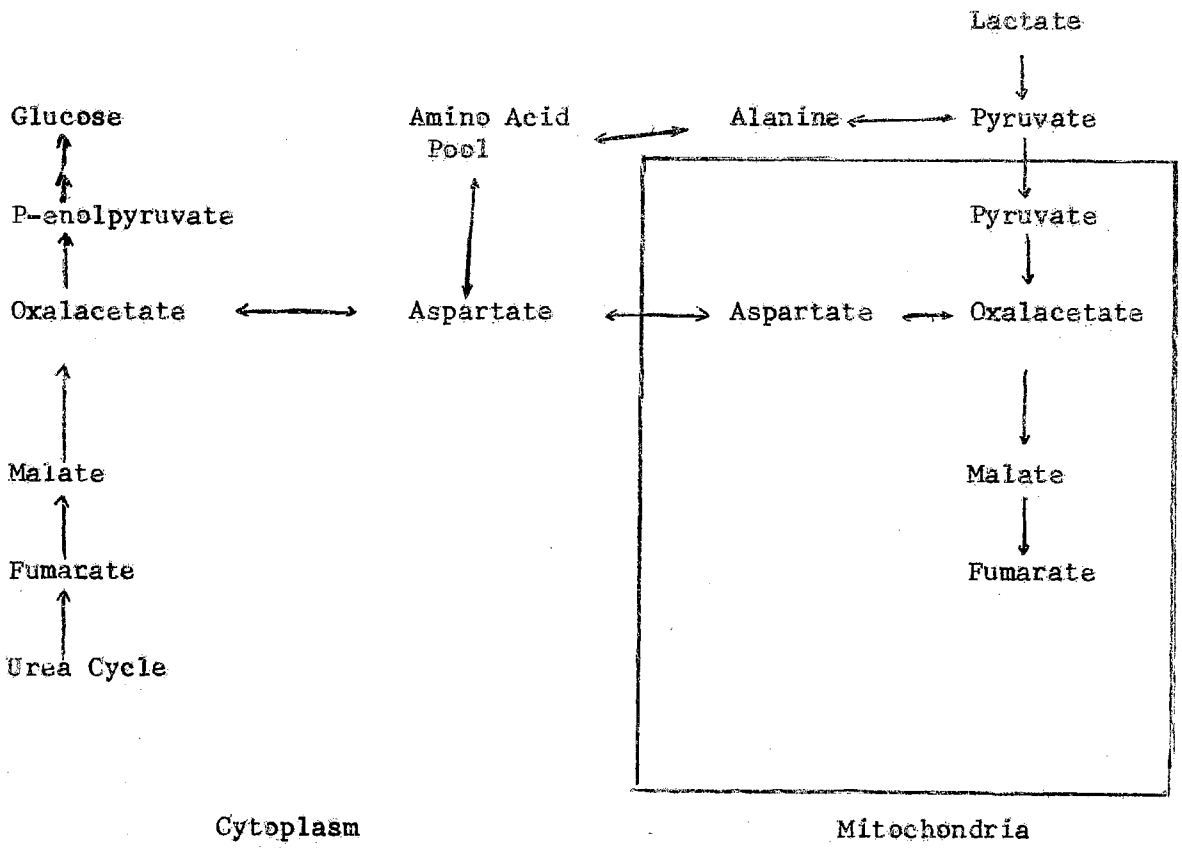

Figure 2. Proposed Scheme for P-enolpyruvate Formation in the Rat Liver from Decarboxylic and Amino Acids.

proposed by Shrage and Lardy (41) for the formation of P-enolpyruvate in rat liver from dicarboxylic and amino acids.

As illustrated in Figure 2, the compartmentalization of enzymes, cofactors, and substrates is a very important factor in the regulation of the metabolism of pyruvate.

The requirement of pyruvate carboxylase for ATP and the inhibition of this enzyme by ADP (26) suggests that the ratio of ATP to ADP in the mitochondria may be an important factor in the regulation of the metabolic fate of pyruvate. Berry (47) has shown that in isolated liver cells and mitochondria ADP, at concentrations found *in vivo* (48), can stimulate the rate of pyruvate oxidation and suppress pyruvate carboxylation. Walter, Paetkau, and Lardy have also shown that ADP inhibits pyruvate carboxylase (49).

The obligatory requirement for acetyl-CoA by pyruvate carboxylase has prompted some investigators to suggest that cellular acetyl-CoA levels may be a controlling factor in the rate of formation of oxalacetate from pyruvate (3,36). The ability of acetoacetate to stimulate gluconeogenesis in kidney slices in the presence of lactate is, according to Krebs (3), probably the result of acetyl-CoA (from acetoacetate) activating pyruvate carboxylase.

A possible control site in the conversion of lactate to triose phosphate has been demonstrated by Exton and Park (50). They have concluded from studies of lactate metabolism in perfused rat livers that the rate-limiting step in gluconeogenesis from lactate is the formation of triose phosphate from pyruvate. Gevers and Krebs' work (51) with pigeon liver homogenates agrees with and extends this con-

cept by showing that AMP inhibits the conversion of pyruvate to P-enol-pyruvate. They suggested that the enzyme which is inhibited is P-enol-pyruvate carboxykinase. Holten and Nordlie (5) have observed an inhibition of guinea pig liver mitochondrial P-enolpyruvate carboxykinase by AMP. It is also noteworthy that while P-enolpyruvate carboxykinase activity is increased following glucocorticoid administration, in fasting and in diabetes (43), pyruvate carboxylase activity is probably unaffected (41).

The inhibition of pyruvate oxidation by free fatty acids and ketone bodies in rat diaphragm (53), in perfused guinea pig heart (54), in infused dog liver (55), and in rat liver mitochondria (49) suggests still another mechanism capable of controlling pyruvate metabolism. The mechanism whereby fat oxidation controls pyruvate metabolism is not known. It is possible that the enhanced utilization of fat, by competing for the coenzymes, may interfer with the oxidative decarboxylation of pyruvate. Garland and Randle(56) have shown that acetyl-CoA is a competitive inhibitor, with respect to CoA, of pig heart pyruvate dehydrogenase. They have also shown that in diabetes, starvation, or after perfusion with free fatty acids pig heart acetyl-CoA concentrations are increased. The accumulation of acetyl-CoA in rat liver as a result of diabetes or alimentary fat loading has also been reported (57). However, these authors were unable to find any increase in acetyl-CoA levels after a 48 to 76 hour fast (58).

Changes in levels of oxalacetate have been postulated to control the relative rates of acetyl-CoA incorporation into citrate or acetoacetate (47,48). Evidence supporting this hypothesis is that in very

severe diabetes oxalacetate levels are decreased in the liver (59), and in carefully controlled conditions *in vivo* (47) pyruvate is diverted to ketone bodies because of a lack of oxalacetate; however, it has been shown that oxalacetate levels do not decrease in fasting or light diabetes (58,60,61). The fact that short chain fatty acids are oxidized *in vivo* and *in vitro* at an equal rate in fed, fasted, and diabetic animals indicates that sufficient oxalacetate is available for fatty acid oxidation (54,62,63,64,65). Further evidence against oxalacetate levels controlling acetyl-CoA metabolism is provided by the work of Shepherd *et al.* (66). They have shown that in rat liver mitochondria the relative rates of conversion of acetyl-CoA into citrate or acetoacetate are independent of the measured oxalacetate concentrations. They propose that oxalacetate is compartmentalized in the mitochondria.

Friedmann *et al.* (67) state, "much evidence is now available to support the view, long controversial, that hepatic gluconeogenesis is under the control of insulin." Although this statement appears to be inharmonious with the well established role of the glucocorticoid hormones, there are considerable data available showing that compared to normal fasted rats, adrenalectomized rats are capable of converting similar amounts of labeled pyruvate, CO_2, malate, and lactate into glucose and glycogen (68,69,70,71). Weber *et al.* (72), upon finding that the induction of enzymatic activity by glucocorticosteroids was suppressed by insulin, suggested that gluconeogenesis is regulated by a balance between the adrenal steroids as inducers and insulin as a suppressor of gluconeogenic enzymes. Friedmann *et al.* (67), nevertheless, feel that the glucocorticoids probably act as releasers of insulin

inhibition rather than as initiators of gluconeogenic activity.

Labeling patterns *in vivo* in liver glutamate have been determined in order to evaluate the effects of fasting, insulin, alloxan diabetes, and thiamine deficiency on pyruvate metabolism (28). When pyruvate-2-^{14}C and alanine-2-^{14}C are metabolized via acetyl-CoA, the resulting carboxyl-labeled acetyl-CoA will be incorporated into carbons 1 and 5 of glutamate (27,28). If pyruvate-2-^{14}C and alanine-2-^{14}C are metabolized via oxalacetate and then to α-ketoglutarate, the resulting glutamate will be labeled in carbons 2 and 3. It has been shown by Koeppe *et al.* (28) using pyruvate-2-^{14}C and Freedman and Graff (29) using alanine 2-^{14}C that liver glutamate obtained from fasted rats has 2 to 3 percent of its total labeling in carbon 5. Glutamate isolated from the livers of fed rats has 30 to 40 percent of its total labeling in carbon 5. Assuming that the dicarboxylic acids and acetyl-CoA pools are not drastically changed, and neglecting the possibility that ^{14}C in the dicarboxylic acid pool may result from exchange reactions with pyruvate, the low amount of labeling in carbon 5 and the corresponding increase in carbons 2 and 3 of glutamate as a result of fasting probably indicate a change in pyruvate metabolism from the acetyl-CoA route to the oxalacetate route. This interpretation agrees with the now well known observation that there is a high incorporation of pyruvate into blood glucose of fasted and diabetic rats, and a relatively low incorporation of this compound into the blood glucose of fed rats (67). Koeppe et al. (28) obtained a typical fasting glutamate labeling pattern in short term diabetes; however, in long term diabetes the labeling patterns observed in liver glutamate resembled those

found in fed rats. This difference is yet to be explained. Whereas Gubler (73) showed a decrease in pyruvate decarboxylation in thiamine deprived animals, Koeppe et al. (28) were unable to obtain a decrease in labeling of carbon 5 of glutamate following pyruvate-2-^{14}C administration to thiamine deficient animals. These data and unpublished results indicate that severe thiamine deficiency does not alter the percentage of pyruvate metabolized via acetyl-CoA compared to that via oxalacetate. Gubler (73), in agreement with the results obtained by Koeppe (28), states that the decrease in oxidative decarboxylation of pyruvate is not sufficient to account for the observed effects of thiamine deficiency.

There has been considerable research done showing that pyruvate is incorporated more rapidly into glucose in fasted animals than in fed animals. The increase in fatty acid oxidation with a corresponding decrease in fatty acid synthesis is also a well-known phenomenon. Labeling patterns in glutamate following the administration of pyruvate-2-^{14}C are known to be profoundly affected by nutritional conditions. However, it is not known whether these labeling patterns found in glutamate are indicative of the relative amounts of pyruvate converted to acetate and oxalacetate, or whether they reflect changes in the sizes of certain metabolic pools. In view of the above, the objectives of this study were:

1. To determine if there is a direct relationship between labeling patterns in glutamate and the route of pyruvate metabolism.

2. To determine if mammals other than rats show the same differences in "fed" and "fasted" glutamate labeling patterns following the

administration of pyruvate-2-^{14}C.

3. To determine if the activity of liver pyruvate dehydrogenase changes in fed and fasted rats.

4. To develop a system which will enable one to study the labeling patterns in glutamate *in vitro*.

CHAPTER II

EXPERIMENTAL

Source of Animals

Young male albino rats (130 to 300 gm) obtained from the Holtzmann Rat Company were used in all of the *in vivo* and *in vitro* rat experiments. The other mammals used in the *in vivo* survey of pyruvate metabolism were of unknown strain. The guinea pigs and hampsters were purchased from a pet shop in Stillwater, Oklahoma. The rabbits were obtained from various Stillwater farmers, and the albino mice were obtained from the Department of Microbiology at Oklahoma State University.

A stock Purina diet and water were fed *ad libitum* until the various experimental conditions were imposed. All animals were killed by decapitation early in the morning to insure that the animals would still be in a fed condition. Animals were considered fed if their stomachs still contained food. Animals labeled as fasted were without food for 48 hours, unless stated otherwise.

Tissue Preparations

Liver mitochondria used in the ferricyanide spectrophotometric assays were prepared essentially according to the method of Schneider and Hogeboom (74). After the animals were sacrificed and bled, the livers were quickly removed and chilled in beakers containing ice-cold

0.25 M sucrose. After cooling, the livers were blotted to remove excess sucrose solution, and then weighed and homogenized in a Potter-Elvehjem glass homogenizer with a plastic (Kel-F) pestle. Cold 0.25 M sucrose solution was used as the homogenizing medium. The final volume of the homogenate in ml was 10 times the wet weight in gm of the liver used. Liver homogenates prepared in this manner were used for the studies involving pyruvate-2-^{14}C incorporation into glutamate and aspartate. The homogenates were centrifuged at 600 x g for 10 minutes to remove nuclei and cell debris. The remaining supernatant solution was centrifuged at 8500 x g for 10 minutes. The sedimented mitochondria were washed by resuspending them in a volume of 0.25 M sucrose equal to nine times the wet weight of the liver. They were homogenized and recentrifuged at 8500 x g for 10 minutes. The pellet of mitochondria was resuspended by homogenization with a Potter-Elvehjem homogenizer. Enough 0.25 M sucrose was then added to make the final volume in milliliters equal to the wet weight, in grams, of the original tissue used. All isolation procedures were carried out in a cold room at 4°. A 1:5 dilution of the original mitochondrial suspension was used for the ferricyanide spectrophotometric assay of pyruvate oxidation.

Mitochondria used in the pyruvate-1-^{14}C studies and in the O_2 uptake studies were prepared according to the method described by Hogeboom (75). The livers were chilled immediately after removal by immersion in 0.25 M sucrose at 0° and then were blotted and weighed. The liver was minced with scissors and homogenized for 2 minutes in a volume of cold 0.25 M sucrose equal to nine times the weight, in grams, of the tissue homogenized. This homogenate was carefully layered over

an equal amount of 0.34 M sucrose in a lusteroid centrifuge tube. After centrifuging for 10 minutes at 700 x g, the supernatant solution was removed and recentrifuged at 5000 x g for 10 minutes. The supernatant solution, together with a pink, partially sedimented layer of particles, was decanted away from the firmly packed pellet of mitochondria. This pellet was resuspended in a volume of 0.25 M sucrose equal to one-half of the original volume used and centrifuged at 2400 x g for 10 minutes. The resulting pellet of mitochondria was resuspended in 0.25 M sucrose by homogenization, and 0.25 M sucrose was added to make the final volume in ml equal to the weight in gm of the amount of original tissue used. Suitable aliquots were used for the O_2 uptake and pyruvate-1-^{14}C decarboxylation studies.

Liver slices were prepared with a Stadie-Riggs slicer (0.5 mm). A lobe of liver, which had been previously chilled in either Krebs Ringer Phosphate (KRP) or Krebs Ringer Bicarbonate (KRB) buffer, was cut to an appropriate size, circa 2 cm^2, and placed in the slicer. Approximately 6 to 10 slices were placed in a tared flask containing 12 ml of either KRP or KRB buffer. Each flask, cooled in ice, contained slices from a different lobe of the liver. From 2.0 to 2.5 μc of pyruvate-2-^{14}C were added to the flasks, and the flasks quickly weighed. They were then placed in a 37° shaking water bath, aerated, and shaken at a rate of 100 oscillations per minute for the duration of the experiment. When KRB was the buffer, the slices were aerated with a 95:5 mixture of O_2 and CO_2; when the buffer was KRP, the aerating gas was O_2.

Assays in Vitro

Pyruvate Dehydrogenase Studies

The oxidation of pyruvate was measured using three different assay techniques: 1. A spectrophotometric method in which potassium ferricyanide acted as the ultimate electron acceptor. 2. A standard manometric method. 3. The rate of decarboxylation of pyruvate was followed by measuring the formation of $^{14}CO_2$ from pyruvate-1-^{14}C.

Spectrophotometric Assay. The spectrophotometric method used was essentially that of Gubler's (73). The reduction of ferricyanide was measured with a Beckman Model D U spectrophotometer by following the change in absorbance at 420 mμ. In order to reduce the effects of variation in light transmission caused by variation in turbidity of the samples, the samples were read through the frosted sides of the glass cuvettes. Readings were taken at 5 minute intervals. Before each reading was taken, the contents of the cuvette were stirred with a teflon stick to resuspend any mitochondria that had settled to the bottom of the cuvette. A blank containing only mitochondria and ferricyanide was used in each experiment to cancel out any changes in absorbance resulting from the reduction of ferricyanide by endogenous substrate or from the swelling of the mitochondria. When the endogenous rate was high, the enzyme preparation was allowed to preincubate with a few drops of ferricyanide solution for 15 to 20 minutes.

The incubation mixture consisted of the following reagents: K_2HPO_4 buffer, 75 μ moles (0.15 M pH 7.4); $MgSO_4$, 20 μmoles; versene, 2 μ moles; ATP, 6 μ moles; pyruvate, 20 to 40 μ moles; 0.2 to

0.4 ml of a 1:5 dilution of mitochondrial suspension in 0.25 M sucrose; and 0.25 M sucrose to make the volume 3 ml. To this was added 0.7 ml of 6.66 mM $K_3Fe(CN)_6$.

Total nitrogen was determined with Nessler's reagent as described by Pelies and Van Slyke (76). Nessler's reagent was prepared according to the method of Bock and Benedick (77). Seventy gm KI and 100 gm HgI_2 were dissolved in 400 ml H_2O. A second solution containing 100 gm NaOH in 500 ml of H_2O was added to the KI-HgI_2 solution. After cooling, this stock solution was placed in a brown pyrex bottle and stored in a dark place. A suitable aliquot of the mitochondrial preparation (0.2 to 0.4 ml) was digested in a micro-Kjeldahl flask using 1 ml of 50 percent H_2SO_4 and 1 ml of saturated $KClO_4$ (78). After digestion the solution was cooled and 30 ml of H_2O were added to the flask. To one volume of stock Nessler's solution was added 2 volumes of 10 percent NaOH. Fifteen ml of this diluted reagent were added to the contents of the digestion flask. The resulting solution was diluted to a volume of 50 ml with H_2O, and after 5 minutes the samples were read at 480 mμ in a Coleman Jr. spectrophotometer.

Oxygen Uptake Assay. A standard Warburg technique was used in the oxygen uptake studies (79). The contents of the Warburg flasks were as follows: ATP, 6 μ moles; phosphate buffer, 40 μmoles (0.15 M pH 7.4); $MgSO_4$, 15 μmoles; pyruvate, 30 μmoles; 0.3 to 0.4 ml mitochondrial suspension; and 0.25 M sucrose to a final volume of 3.0 ml.

Pyruvate-1-^{14}C Decarboxylation Assay. The decarboxylation of pyruvate-1-^{14}C was conducted in a 25 ml Erlenmeyer flask with a fixed center well (6 mm x 2.5 cm). The method used to trap the $^{14}CO_2$ was similar to that of Snyder and Godfrey (80), except they used a Warburg flask. The reaction medium was the same as that used in the ferricyanide studies less ferricyanide. The reaction vessel was kept in ice during the time the reagents were added. After the standard reaction mixture was placed in the flask, 10 to 20 μ1 of pyruvate-1-^{14}C (2.6 to 5.2 x 10^5 cpm) were added with a gas chromatographic injection syringe. After the mitochondria were placed in the flask, it was sealed with a serum stopper and placed in a 37° shaker water bath. At the end of one hour, or at appropriate time intervals, 0.5 ml Hyamine was injected through the rubber stopper into the center well. Then 0.3 ml of 6 N H_2SO_4 was injected into the reaction medium to stop the reaction and to release the $^{14}CO_2$. After 3 hours of shaking to insure complete recovery of the $^{14}CO_2$ that was in the reaction medium, the Hyamine was removed with a Pasteur pipette and placed in a scintillation vial. The center well was washed five to six times with the scintillation fluid. The scintillation cocktail consisted of 0.4 percent PPO, 0.01 percent POPOP, and toluene. A blank flask containing all reagents except mitochondria was used to measure the amount of $^{14}CO_2$ produced by the acid-catalyzed decarboxylation of pyruvate. In trial runs using $NaH^{14}CO_3$, it was shown that 97 to 98 percent of the added $^{14}CO_2$ was recovered from the center well after three hours of shaking in a water bath at 37°. In the oxygen uptake and pyruvate-1-^{14}C studies liver mitochondria were isolated from fed

and fasted animals simultaneously and paired experiments were run using mitochondria from fed and fasted rats.

Pyruvate-2-^{14}C Incorporation In Vitro Into Glutamate and Aspartate

The incorporation of pyruvate-2-^{14}C into the glutamate and aspartate of liver homogenates and slices was determined under a variety of conditions. Homogenates were divided into two equal portions to permit duplicate runs to be made. In all of the homogenate experiments the final molarity of the reagents were: KCl, 67 mM; phosphate buffer, 10 mM (pH 7.4); $MgSO_4$, 3.3 mM; ATP, 6 μ moles; pyruvate, 40 μ moles; ctyochrome C, 6.2 mg. The incubation period varied from fifteen to sixty minutes and was conducted in a 37° shaking water bath.

The buffers used in the slice experiments were either KRP or KRB. KRP minus Ca++ was used as the incubation medium for animals 15 and 16. Neither pyruvate nor ATP was added to any of the reaction vessels, and the time of the experiments was shortened to 6 minutes. The experiments were terminated by adding a volume of 0.66 N perchloric acid equal to 2 x the wet weight of tissue used and homogenizing with a Potter-Elvehjem homogenizer. The resulting solution was centrifuged and the sedimented protein was rehomogenized in an equal volume of 0.33 N perchloric acid. The supernatant solutions were combined and the pH adjusted to 7 with 2 N KOH. After standing overnight in the refrigerator, the solution was centrifuged and the supernatant

solution was placed on a 25 x 1.5 cm Dowex-1-acetate column. Glutamate and aspartate were eluted from the column with 0.5 N acetic acid as described by O'Neal (81). The amount of glutamate and aspartate eluted from the column was determined by the ninhydrin method of Rosen (82) as modified by O'Neal (81). An aliquot of the eluted amino acids was counted in a Packard scintillation counter. Approximately 0.5 to 1 gm of cold glutamate and aspartate were added as carrier to the pooled fractions of glutamate and aspartate, respectively, and the amino acids were recrystallized as described by O'Neal (81). The ^{14}C content of carbon 5 of glutamate was determined with a Schmidt degradation as described by Hahn (83). The amounts of activity in carbons 1 of glutamate and 1 and 4 of aspartate were determined by decarboxylation using ninhydrin method of Van Slyke as described in Hahn's thesis (83). All radioactive determinations in the degradation studies were made with a Cary vibrating reed electrometer.

During the isolation of aspartate and glutamate from the Dowex-1-acetate column, an unknown amino acid preceded glutamate off of the column. The identity of this amino acid has not yet been determined.

A summary of the labeling experiments in vitro is given in Table I.

Labeling Experiments in Vivo

Long Term Experiments

In all of the long term experiments the animals were allowed to metabolize the pyruvate-2-^{14}C, which had been given by intraperitoneal injection, for a period of 2 hours. At the end of this period the animals were sacrificed, and the liver and muscle protein powders

TABLE I

SUMMARY OF THE LABELING EXPERIMENTS IN VITRO

Exp. No.	(gm) Rat Wt.	Gm. of Liver Used	(hr) Time Fasted	Tissue Preparation	Incubation Medium	Amount of Pyruvate-2-^{14}C Used (μC)	Substrate Added	μmole of Substrate	Time of Incubation (min)
2a	243	5.5	0	Homogenate	K_2HPO_4	5	K-pyruvate	40	15
2b		5.5	0	Homogenate	K_2HPO_4	5	K-pyruvate	40	30
4a	183	2.5	48	Homogenate	K_2HPO_4	5	K-pyruvate	40	15
4b		2.5	48	Homogenate	K_2HPO_4	5	K-pyruvate	40	30
8a	278	4.3	48	Homogenate	K_2HPO_4	2.5	Cytochrome C	6.3[Z]	30
8b		4.3	48	Homogenate	K_2HPO_4	2.5	Cytochrome C	6.3[Z]	60
9a	223	3.2	48	Homogenate	K_2HPO_4	2.5	Cytochrome C	6.3[Z]	60
9b		3.2	48	Homogenate	K_2HPO_4	2.5	Octanoate	40	60
10	280	1.32	48	Slice	KRB[X]	1.25			30
11a	243	0.78	48	Slice	KRB	2.5			6
11b		0.85	48	Slice	KRB	2.5	Fumarate	40	6
12a	205	1	0	Slice	KRB	2.5			6
12b		0.92	0	Slice	KRB	2.5			6
13	250	0.74	48	Slice	KRB	2.5			6
14a	273	0.59	48	Slice	KRP[Y]	2.5			1
14b		0.68	48	Slice	KRP	2.5			6
15a	316	1.16	48	Slice	KRP-Ca++	2.5			0.3
15b		0.95	48	Slice	KRP-Ca++	2.5			6
16a	265	1.21	0	Slice	KRP-Ca++	2.5			0.8
16b		1.85	0	Slice	KRP-Ca++	2.5			6

X refers to Krebs Ringer Bicarbonate

Y refers to Krebs Ringer Phosphate

Z concentration of cytochrome C is in mg.

prepared by extraction of the respective tissues with 10 percent trichloracetic acid, acetone, and ether as described by Hill et al. (37) Cheung (84). After hydrolysis with 6 N HCl and removal of the aromatic acids with activated charcoal, the protein hydrolysates were placed on a Dowex-1-acetate column, and glutamate and aspartate were isolated as described by Cheung (84).

In the hormone studies a single dose of hormone, either hydrocortisone, 2.5 mg/100 gm of body weight, or 9-α-fluoroprednisolone, 3 mg/100 gm body weight was injected intraperitoneally into fed animals 4 to 24 hours prior to the injection of radioactive pyruvate.

Rat 253 was fed a commercial high fat diet containing 45 percent fat for a period of 5 days before the injection of pyruvate-2-^{14}C. The diet was obtained from Nutritional Biochemicals Incorporated. In Table II the changes in weight of this rat and the amount of glycogen isolated from the liver are compared to the values obtained with fed and fasted rats.

TABLE II

LIVER WEIGHT AND GLYCOGEN OF NORMAL, FASTED, AND FAT FED RATS

	Original Weight gm	Final Weight gm	Liver Weight gm	Liver Glycogen gm
R253	163	184	8	0.38
Fed Rat	230	230	11.5	0.46
Fasted Rat	359	314	7.4	0.06

Short Term Experiments

In short term experiments the animals were allowed to metabolize

the intraperitoneally injected radioactive compound for a period of 6 to 18 minutes. They were then sacrificed, and the soluble amino acids, glutamate and aspartate, were isolated from the liver and degraded as described previously.

Acetoacetate and β-hydroxybutyrate were isolated from the perchloric extract of liver by converting them to their mercury-acetone complex with Deniges' reagent (85). Three hundred to 400 mg of carrier acetoacetate were added to the perchloric acid extract, which was kept ice cold. Ten ml of 50 percent sulfuric acid and 35 ml of 10 percent mercuric sulfate were added to the extract, and the entire solution placed in a 250 ml round bottom flask. The mixture was heated to boiling, treated with 8 ml of 5 percent potassium dichromate solution to oxidize the β-hydroxybutyrate to acetoacetate, and heated under reflux for 90 minutes. At the end of the refluxing period the solution was cooled to room temperature and the yellowish-orange precipitate was removed by filtration and dried at 110° for one hour. This precipitate was recrystallized by steam distilling it from 2 N HCl into a 250 ml round bottom flask containing 35 ml of 10 percent mercuric sulfate and 10 ml of 50 percent sulfuric acid. This mixture was heated for 90 minutes under reflux, and the mercury-acetone complex removed by filtration and dried at 110° for one hour.

Table III gives a summary of the incorporation studies *in vivo*.

Stability of Pyruvate-2-^{14}C

The instability of pyruvate in aqueous solution is a problem that is receiving considerable attention (86,87). Pyruvate-2-^{14}C stored

TABLE III

SUMMARY OF THE INCORPORATION STUDIES IN VIVO

Animal Number	Animal Wt.(gm)	Liver Wt.(gm)	Carcass Wt.(gm)	Time Fasted (hrs)	Isotope Injected Intraperitoneally	μC Inj.	Time of Exp. (min)
R246[a,b]	256	10.8	115	0	Pyruvate-2-^{14}C	20	120
R248[b]	144	6.8	51	0	Pyruvate-2-^{14}C	21	120
R249[c]	189	8.5	74	0	Pyruvate-2-^{14}C	22.5	120
R250	188	9.1	73	0	Pyruvate-2-^{14}C	22.5	120
R253	184	8	82	0[e]	Pyruvate-2-^{14}C	35	120
R304	160	3		144	Pyruvate-2-^{14}C	20	12
R263	182	7.6		0	Pyruvate-2-^{14}C	25	12
R265	191	8.2		0	Pyruvate-2-^{14}C	10	6
R266	201	8.9		0	Pyruvate-2-^{14}C	10	18
R264	173	4.6		48	Pyruvate-2-^{14}C	25	12
R277	270	8.2		48	Pyruvate-2-^{14}C	10	6
R278	255	6.9		48	Pyruvate-2-^{14}C	10	18
R279	215	5.6		48	Pyruvate-2-^{14}C	10	10
R285	243	6		48	Pyruvate-2-^{14}C	21.3	12
R286	272	9.7		0	Pyruvate-2-^{14}C	21.3	12
R298	294	11.8		0	Pyruvate-1-^{14}C	20	12
R299	255	8.3		48	Pyruvate-1-^{14}C	20	12
R300	281	8.9		0	Pyruvate-1-^{14}C	20	12
R301	225	5.5		48	Pyruvate-1-^{14}C	20	12
R287	209	6.2		48	Butyrate-1-^{14}C	25	12
R296	236	9.1		0	Butyrate-1-^{14}C	25	12
R302	254	10.4		0	Butyrate-1-^{14}C	25	12
R303	207	6.3		48	Butyrate-1-^{14}C	25	12
R297	240	11.7		0	Butyrate-1-^{14}C	25	12
R295	232	5.7		48	Butyrate-1-^{14}C	25	12
M1	37	1.7	13.3	0	Pyruvate-2-^{14}C	12.5	120
M4	40	1.5	17.4	48	Pyruvate-2-^{14}C	20.8	120
G1	576	15.8	222	0	Pyruvate-2-^{14}C	21.5	120
G2	535	13.6	226	48	Pyruvate-2-^{14}C	20	120
Rb1[d]	1364	60.4	109	0	Pyruvate-2-^{14}C	45	120
Rb2	2038	51.3	204	51	Pyruvate-2-^{14}C	41	120
Rb3	1250	31	135	61	Pyruvate-2-^{14}C	50	120
Rb4	1008	30	110	0	Pyruvate-2-^{14}C	40	120
H1	65	2.9	26.3	0	Pyruvate-2-^{14}C	23.5	120
H2	62	2.8	23	0	Pyruvate-2-^{14}C	23.5	120
H3	82	2.7	32	48	Pyruvate-2-^{14}C	29.4	120

a. R refers to rat, M to mouse, G to guinea pig, Rb to rabbit, and H to hampster.
b. Administered hydrocortisone.
c. Administered 9-α-fluoroprednisolone.
d. Rabbits 1, 2, and 3 were injected intraveniously.
e. Rat 253 was fed a high diet for 5 days.

for one week in aqueous solution at -15° loses one-third of its radiopurity (87). Freezing and thawing causes it to deteriorate even more rapidly (87).

Studies were undertaken to determine the stability of pyruvate under the conditions of handling normally used in this laboratory. The purity of pyruvate was determined with Celite chromatography as described by Phares et al. (88). In a typical experiment pyruvate-2-^{14}C obtained from Nuclear Chicago was dissolved in 1 ml of H_2O on 6/1/64, and frozen. On 6/3/64, the sample was melted and 2.5 μc of pyruvate-2-^{14}C plus 35 mg of carrier potassium pyruvate dissolved in 0.3 ml of H_2O were mixed with dry Celite and 0.1 ml of 6 N H_2SO_4 in an ice-cold beaker. The resulting mixture was placed on top of a Celite column 33.5 x 1.2 cm. The pyruvate was eluted from the column with 100 ml of CB-5 (chloroform-butanol 95:5), 100 ml of CB-10, 100 ml of CB-20, and 100 ml CB-40, respectively. The flow rate of the column was 15 to 19 drops per minute and the size of the fractions collected was 3 ml. Before the fractions were titrated with 0.09 N NaOH, 1 ml of H_2O and 2 drops of bromthymol blue were placed in each tube. After titration, 0.5 ml of aqueous solution was taken from each tube and spotted on a planchett and counted in a planchett counter to determine its radioactivity.

The results obtained from pyruvate-2-^{14}C treated under different conditions are presented in Figures 3, 4, and 5.

It can be seen from these results that radioactive pyruvate stored as a frozen solution rapidly loses its radiopurity. Because Celite chromatography is a long tedious process, a more rapid method involving

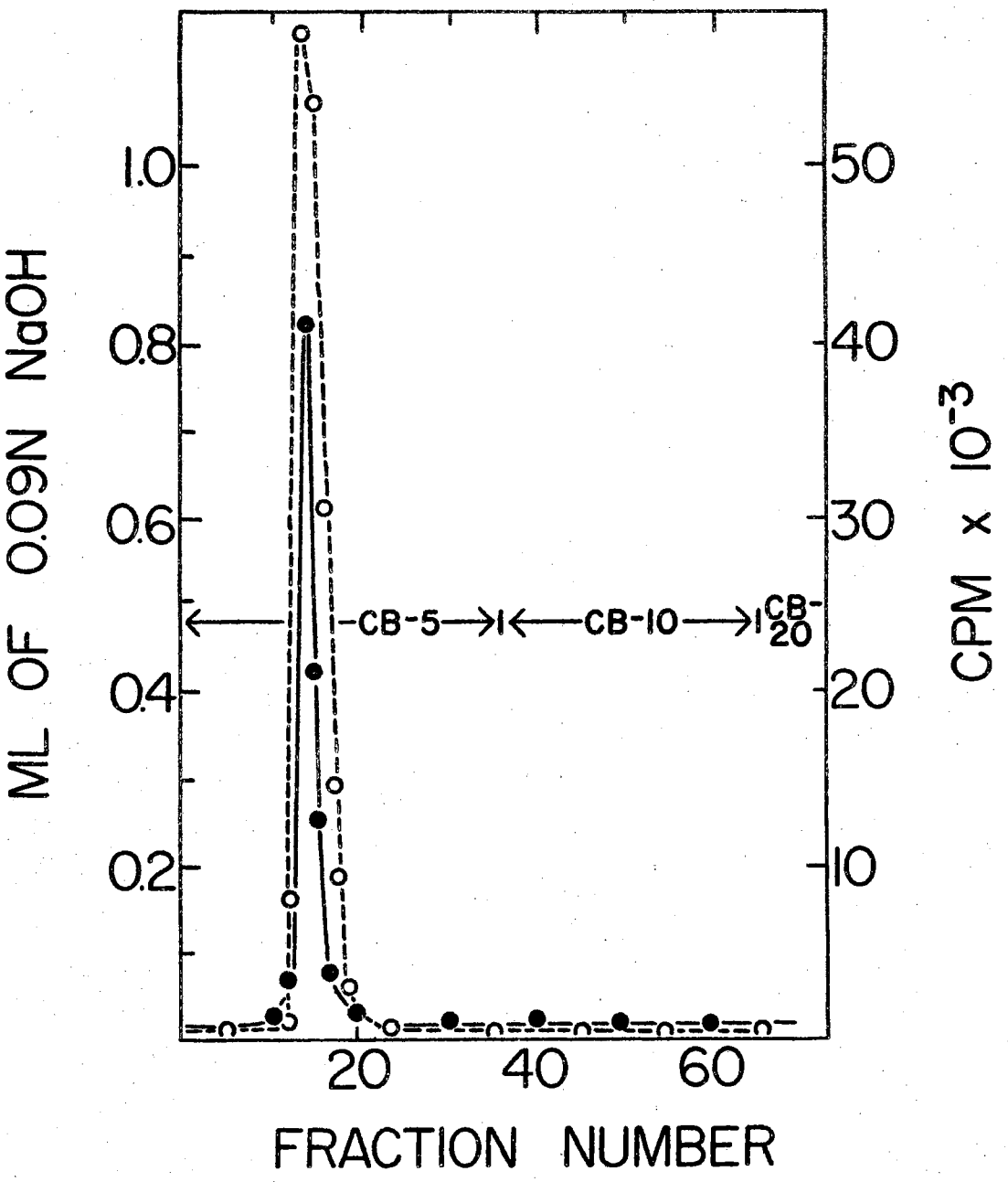

Figure 3. Celite Chromatography of an Aqueous Sample of Sodium Pyruvate-2-^{14}C which had been Stored Frozen for 7 Days.

A sample vial containing 50 μc of pyruvate-2-^{14}C was frozen in 0.5 ml of H_2O on 4/27/64. On 5/4/64, 10 λ of this solution plus 33 mg of potassium pyruvate were chromatographed. 3 ml fractions were collected. ●——●, total ml of NaOH/fraction; ○- - -○, total cpm x 10^{-3}/fraction.

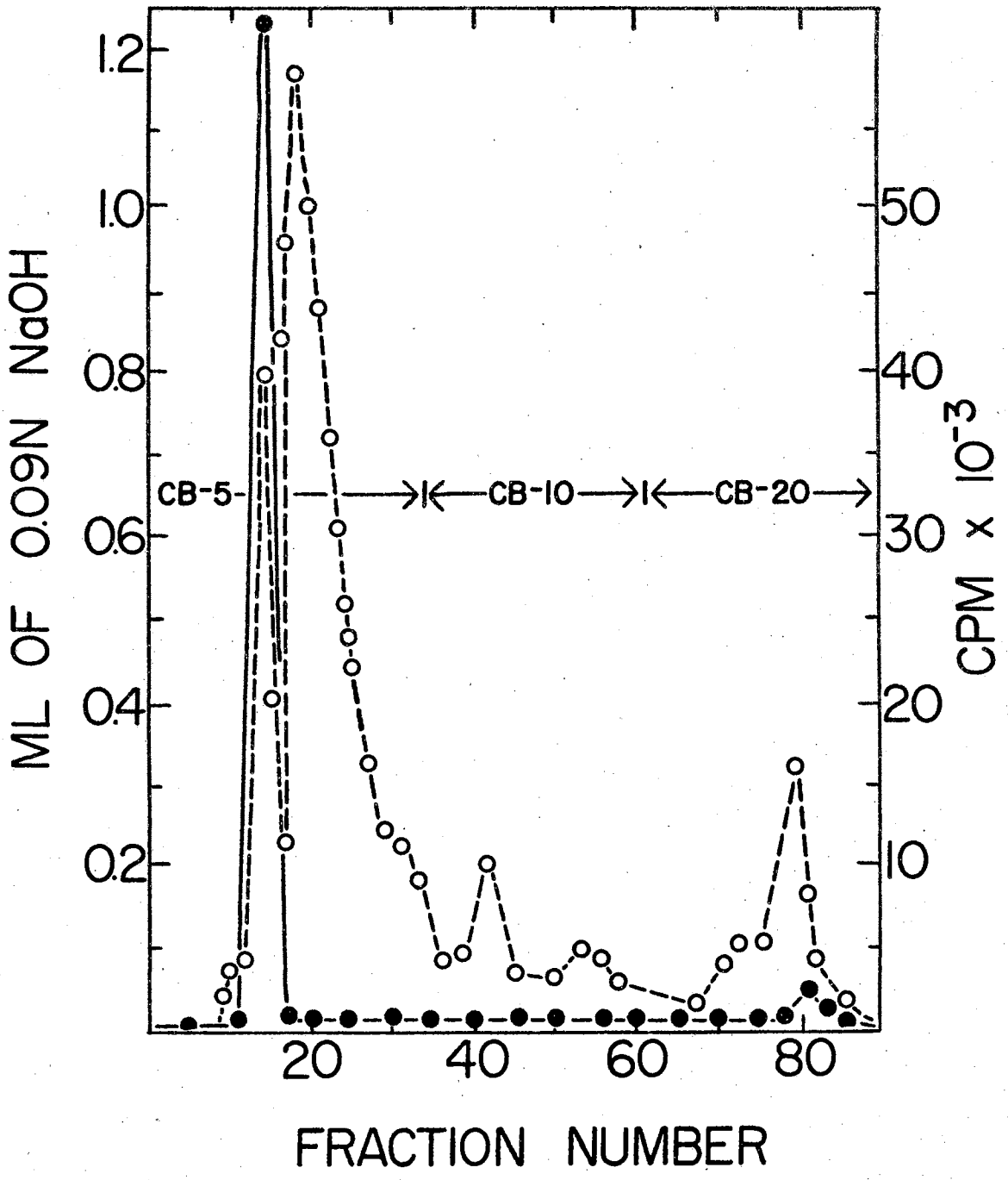

Figure 4. Celite Chromatography of an Aqueous Sample of Sodium Pyruvate-2-^{14}C which had been Stored Frozen for Approximately 3 Months.

A sample vial containing 50 μc of pyruvate-2-^{14}C was frozen in 0.5 ml of H_2O on 1/27/64. On 5/4/64, 10 λ of this solution plus 33 mg of potassium pyruvate were chromatographed. 3 ml fractions were collected. ●——● , total ml of NaOH/fraction; o- - -o , total cpm x 10^{-3}/fraction.

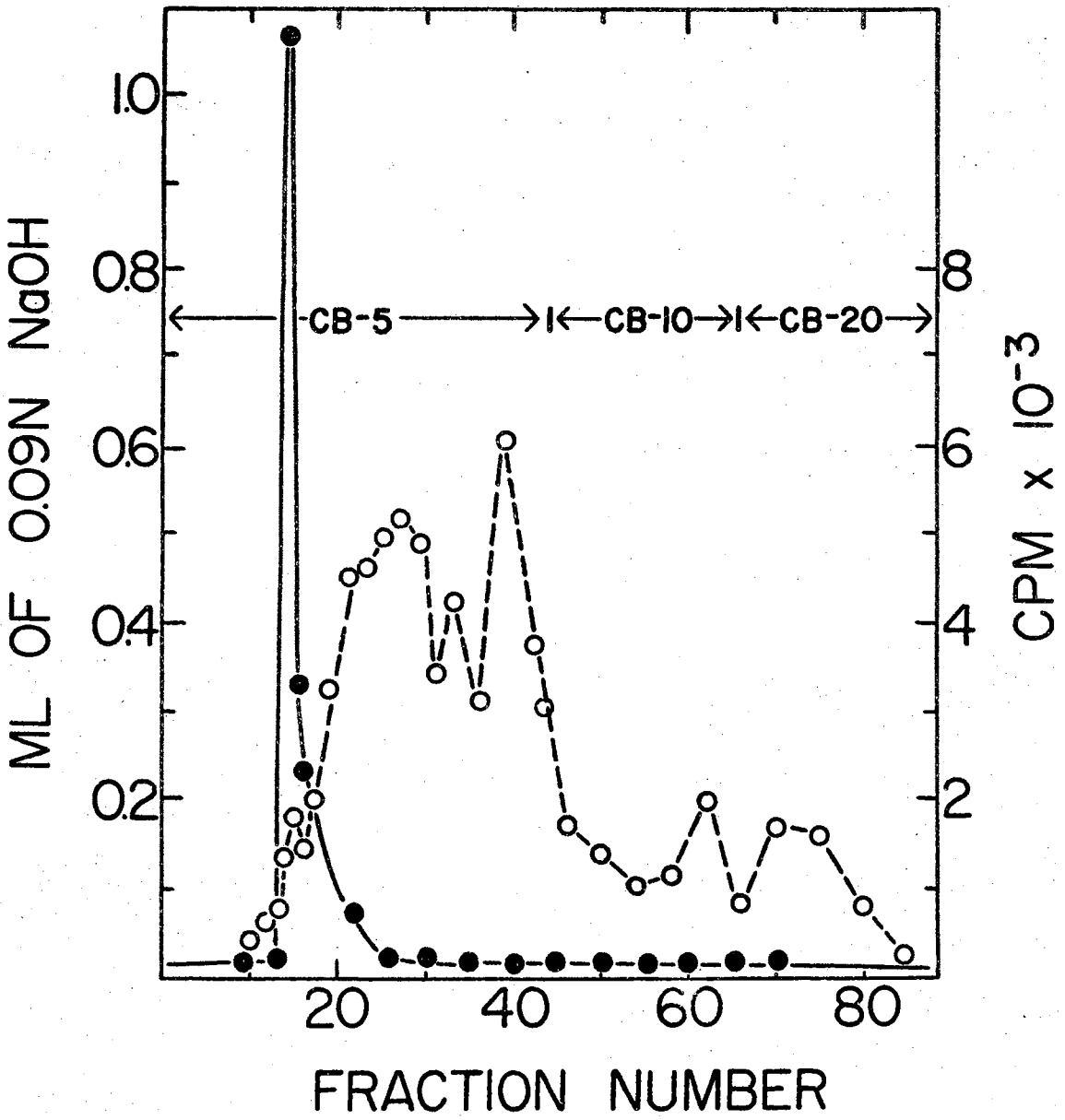

Figure 5. Celite Chromatography of an Aqueous Sample of Sodium Pyruvate-2-^{14}C which had been Alternately Frozen and Thawed 3 Times during a Period of 10 Months.

A sample vial containing 50 μc of pyruvate-2-^{14}C was frozen in 0.5 ml of H_2O on 6/26/64. The sample was thawed and refrozen on 7/3/64, and on 1/4/65. On 4/24/65, 10 λ of this solution plus 33 mg of potassium pyruvate were chromatographed. 3 ml fractions were collected. ●——●, total ml of NaOH/fraction, O- - -O, total cpm x 10^{-3}/fraction.

the chromatography of pyruvate or its 2,4 dinitrophenylhydrazine derivative (DNP-pyruvate) on paper and thin layer plates was attempted. Thin layer chromatography of the potassium and ammonium salts of pyruvate on silica gel G plates or on silica gel G chromagram sheets prepared by Eastman Kodak proved to be unsatisfactory because of too much streaking and because the Rf of pyruvate was very concentration dependant. Figure 6 gives the results of a thin layer chromatogram using EtOH:NH_4OH:H_2O (80:4:16), as the developing solvent. I_2 vapors were used to detect the pyruvate spot.

The pyruvate-2-^{14}C used in chromatograms A and B was prepared as follows: On 8/2/65, a vial of pyruvate-2-^{14}C was opened and diluted to 1 ml. with .9% NaCl. Fractions containing 2.5μc were pipetted into 2 dram vials. The contents of the vials were lyophilized in a dessicator and capped under N_2. On 11/5/65, a vial containing 2.5μc was opened and the pyruvate dissolved in 0.3 ml 50% MeOH. This was divided into 2 equal portions and 1.5 mg of potassium pyruvate was added as carrier to one of the vials. Five λ from each of the vials was spotted on a strip of silica gel G Eastman chromogram and chromatographed. The chromatograms were then placed in a strip counter and the location of the radioactive peaks determined. The position of the carrier pyruvate was determined by I_2 staining. After chromatograms A and B were spotted, 0.15 ml of 0.6 N $HClO_4$ was added to each vial, which was packed in ice. After 30 minutes, 0.15 ml of 0.33 N $HClO_4$ was placed into each of the vials, and the vials were allowed to stand in ice for another 45 minutes. Then 2 drops of bromthymol blue were added to each of the vials and the contents titrated to pH 7 with

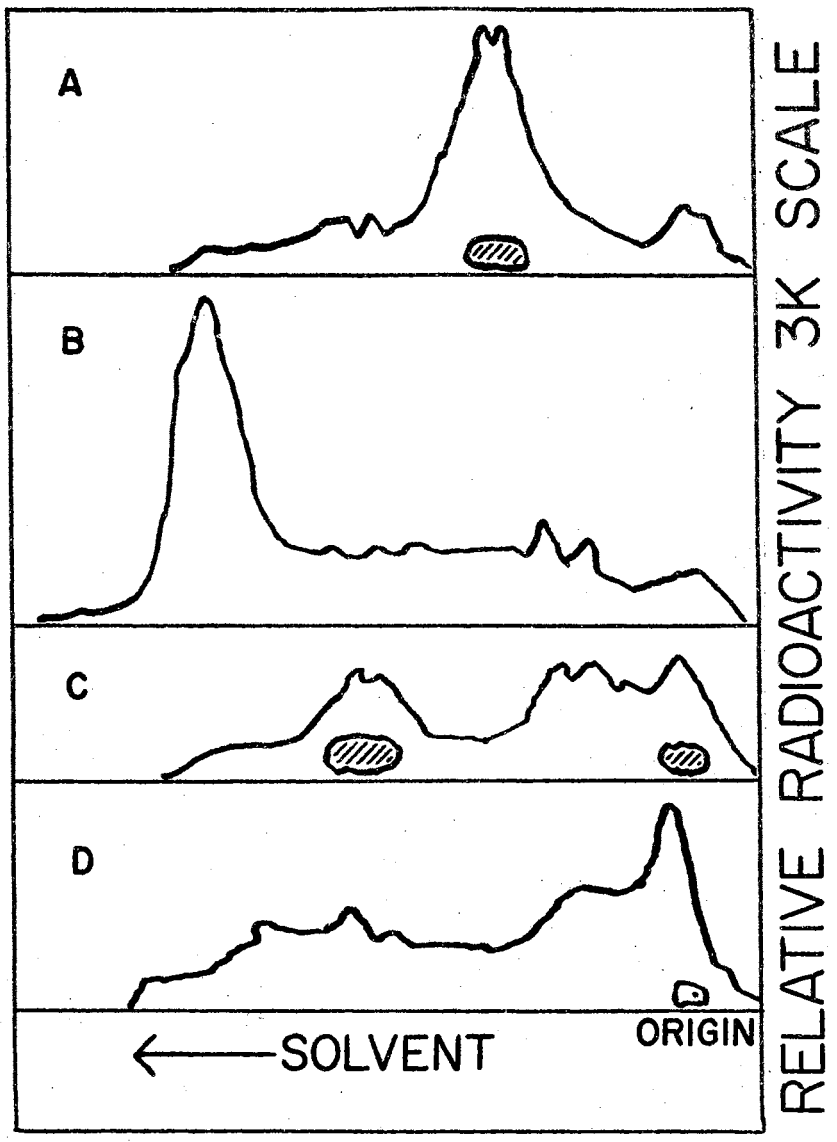

Figure 6. Thin Layer Chromatography of Sodium Pyruvate-2-^{14}C which had been Treated with $HClO_4$.

Iodine stainable material; ——— , relative radioactivity.
Eluting solvent was EtOH:NH_4OH:H_2O (80:4:16)

A. 42 mμc of sodium pyruvate-2-^{14}C plus 0.05 mg potassium pyruvate.
B. 42 mμc of sodium pyruvate-2-^{14}C.
C. 42 mμc of sodium pyruvate-2-^{14}C plus 0.05 mg potassium pyruvate. The samples were treated with $HClO_4$ as described in the text.
D. 42 mμc of sodium pyruvate-2-^{14}C which had been treated with $HClO_4$ as described in the text.

1 N KOH. The vials were allowed to dry on a steam plate. Upon drying the solution became very basic and was retitrated to pH 7 and dried once again on the steam plate. The results obtained when this treated pyruvate was chromatographed are shown in C and D (Figure 6). The slower moving peak, which was detected by its radioactivity, did not develop with I_2 treatment. The spot in chromatogram C that did develop when treated with I_2 vapors is probably pyruvate, which because of its lower concentration has a higher Rf than the pyruvate in the untreated controls. Nonradioactive pyruvate was treated with $HClO_4$ at room temperature in the manner previously described, except, that after neutralization the solution was cooled in ice for 30 minutes and then filtered and lyophilized to dryness. The lyophilized pyruvate produced a chromatographic pattern identical to that obtained with the untreated control.

Thin layer chromatography showed that pyruvate treated with $HClO_4$ was fairly stable, but that heating pyruvate in perchloric acid which had been neutralized with KOH destroyed the pyruvate.

The paper chromatography of the 2,4-dinitrophenylhydrazones of pyruvate proved to be a very desirable method to use to determine the purity of various pyruvate preparations. The method of preparation of the DNP derivaties of pyruvate is a modification of the method of Isherwood and Cruickshank (89).

One ml of an aqueous solution of 3 mM potassium pyruvate was added to 4 ml of 1.5 mM DNPH in 0.2 N HCl in ethanol. After standing for 30 minutes 25 was spoted on Whatman number 1 paper, which had been buffered with 0.1 M phosphate buffer pH 7, and the chromatogram was developed

with a solvent containing n-butanol:ethanol:0.5 N NH_4OH, 7:1:2. Table IV gives the Rf values for various DNP derivatives using ascending and descending chromatography.

TABLE IV

RF'S OF VARIOUS DNP-DERIVATIVES

DNP-Derivative	Type of Chromatography Ascending Rf	Descending Rf
Pyruvate	.43 (.61)	.53 (.72)
Acetaldehyde	.78	.89
Acetone	.92	-
DNPH	.79	.82
Parapyruvate	.19	.19

Pyruvate reacts with DNPH to produce 2 stereo-isomeric hydrazones. The ratio of the 2 hydrazones produced under the reaction condition described is approximately 4:1, the slower moving peak being the more concentrated one.

On 12/2/65, a vial containing 2.5μc of pyruvate-2-^{14}C, which had been lyophilized on 9/17/65, was opened and its contents treated with 0.1 ml H_2O and 0.4 ml of DNPH. After 30 minutes of standing at room temperature, 10λ was spotted on Whatman number 1 phosphate buffered paper. On top of the radioactive DNP-pyruvate spot was placed 100λ of a solution containing approximately 6 x 10^{-5} moles of DNP pyruvate. The pyruvate used to make the carrier DNP-derivative had been allowed to stand at room temperature for 48 hours in order to permit the formation of a considerable amount of parapyruvate. DNP derivatives made from

freshly prepared solutions of potassium pyruvate did not contain any parapyruvate. After the above chromatogram was made, it was developed by descending chromatography. Ascending chromatography is the preferred method as it gives very good separation and the developing time for ascending chromatography is 3.5 hours vs 12 hours for descending chromatography. The results obtained are shown in Figure 7. The chromatogram was monitored with a strip counter. The areas under the two small radioactive peaks represents 3 to 4 percent of the total combined area of all of the peaks.

The preparation of the 2,4-dinitrophenylhydrazine derivatives of pyruvate is an easy and quick method to use for monitoring radioactive samples of pyruvate for impurities. These experiments have also shown that lyophilization of radioactive pyruvate is the preferred method to use for storage.

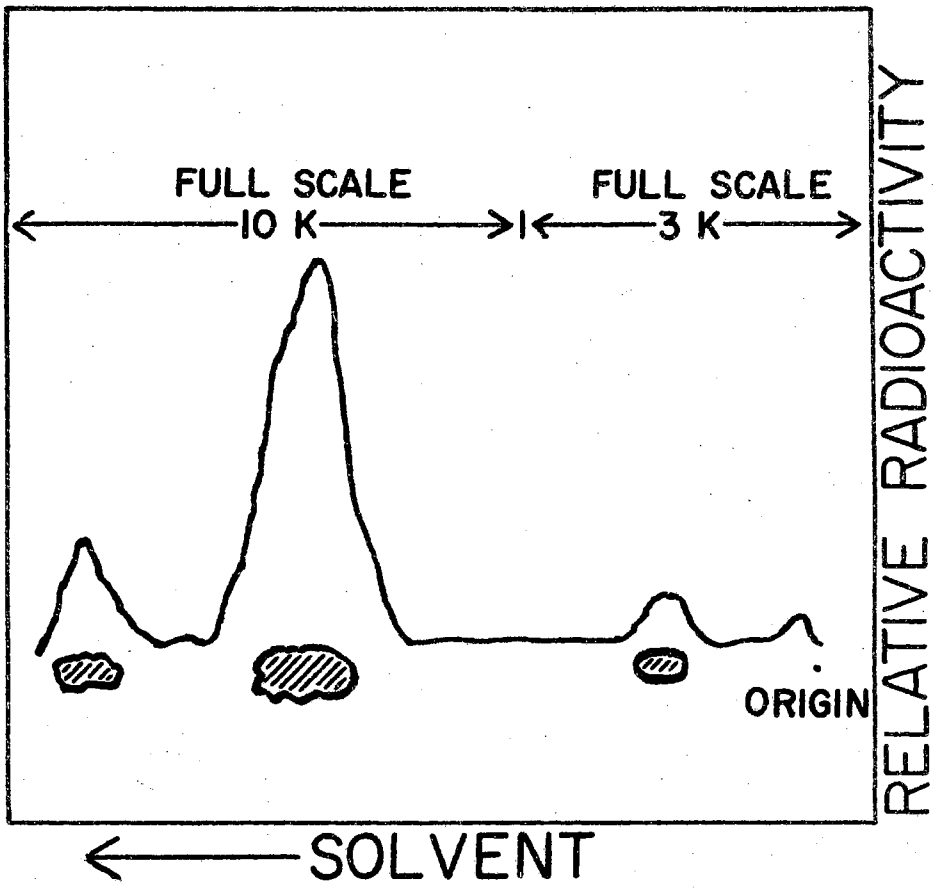

Figure 7. Paper Chromatography of DNP-pyruvate-2-^{14}C.

▨, iodine stainable material; ——— , relative radioactivity. 50 mμc of DNP-pyruvate-2-^{14}C plus 6 x 10^{-5} moles carrier DNP-pyruvate were chromatographed by descending chromatography using n-butanol: EtOH:0.5N:NH_4OH (7:1:2).

CHAPTER III

RESULTS

Pyruvate Dehydrogenase Studies

The results of a series of experiments which were designed to measure the oxidation of pyruvate by mitochondria isolated from fed and fasted rats are given in Tables V and VI. These experiments were undertaken in order to determine if the activity of pyruvate dehydrogenase fluctuated as a function of the nutritional status of the animal.

Table V gives the results obtained when the activity of pyruvate dehydrogenase was measured by the method of Gubler (73). The reduction of ferricyanide was measured as a function of time by following the decrease in absorbance at 420 $m\mu$. Figure 8 shows the linearity of the reaction with time and at two different concentrations of mitochondria. Mitochondria isolated from fed animals oxidized pyruvate at a 27 percent greater rate than did mitochondria from fasted rats.

The O_2 uptake studies were conducted in paired experiments in order to eliminate variations resulting from differences in day to day preparations of mitochondria, time of isolation, etc. Although quite variable, the results suggest that the mitochondria obtained from the fasted liver had a decreased O_2 uptake with respect to the mitochondria isolated from the liver of fed rats (Table V).

TABLE V

EFFECT OF FASTING ON THE OXYGEN UPTAKE AND OXIDATIVE DECARBOXYLATION OF PYRUVATE BY LIVER MITOCHONDRIA

Experiment Number	Hours Fasted	Δ 420/mg N/30 min.	Average Fed	Average Fasted	μ moles O_2/mg N/30 min.	Average Uptake Fed	Average Uptake Fasted
1	0	1.92					
2	0	2.6					
3	0	3.2					
4	0	1.75					
5	0	2.4					
6	0	2.02					
7	48	0.79					
8	48	1.49					
9	48	2.0					
10	48	1.26					
11	48	2.0	2.31	1.51			
12	0				9.74		
13	0				5.58		
14	48				7.55		
15	48				5.16	7.66	6.35

TABLE VI

EFFECT OF FASTING ON THE RATE OF OXIDATION OF PYRUVATE-1-^{14}C TO ACETYL-CoA AND $^{14}CO_2$ BY RAT LIVER MITOCHONDRIA

Experiment Number	K_2HPO_4 Buffer Concentration μ mole	Pyruvate-1-^{14}C Added to the Flask λ	K-Pyruvate Concentration in Flask μ mole	cpm x 10^{-3} in $^{14}CO_2$/mg N/60 min			
				A Normal Rat	B Rat Fasted 48 Hrs	Average of A/B Exp. 1-5	Exp. 6-8
1	40	10[a]	4.4	159.6	102.7	1.34	1.24
2	40	10	4.4	143.7	118.6		
3	40	10	4.4	139.8	105.9		
4[b]	40	10	4.4	65.3	54.5		
5[b]	40	20	4.4	143.8	98.9		
6[b]	5	20	4.4	141.2	111.3		
7	5	10	4.4	142.1	117.5		
8	5	10	4.4	176.9	110.6		

a. 10 λ of pyruvate-1-^{14}C contained 2.6×10^5 cpm

b. In experiments 4, 5, and 6 the pyruvate-1-^{14}C had been stored frozen and the cpm/λ are unknown.

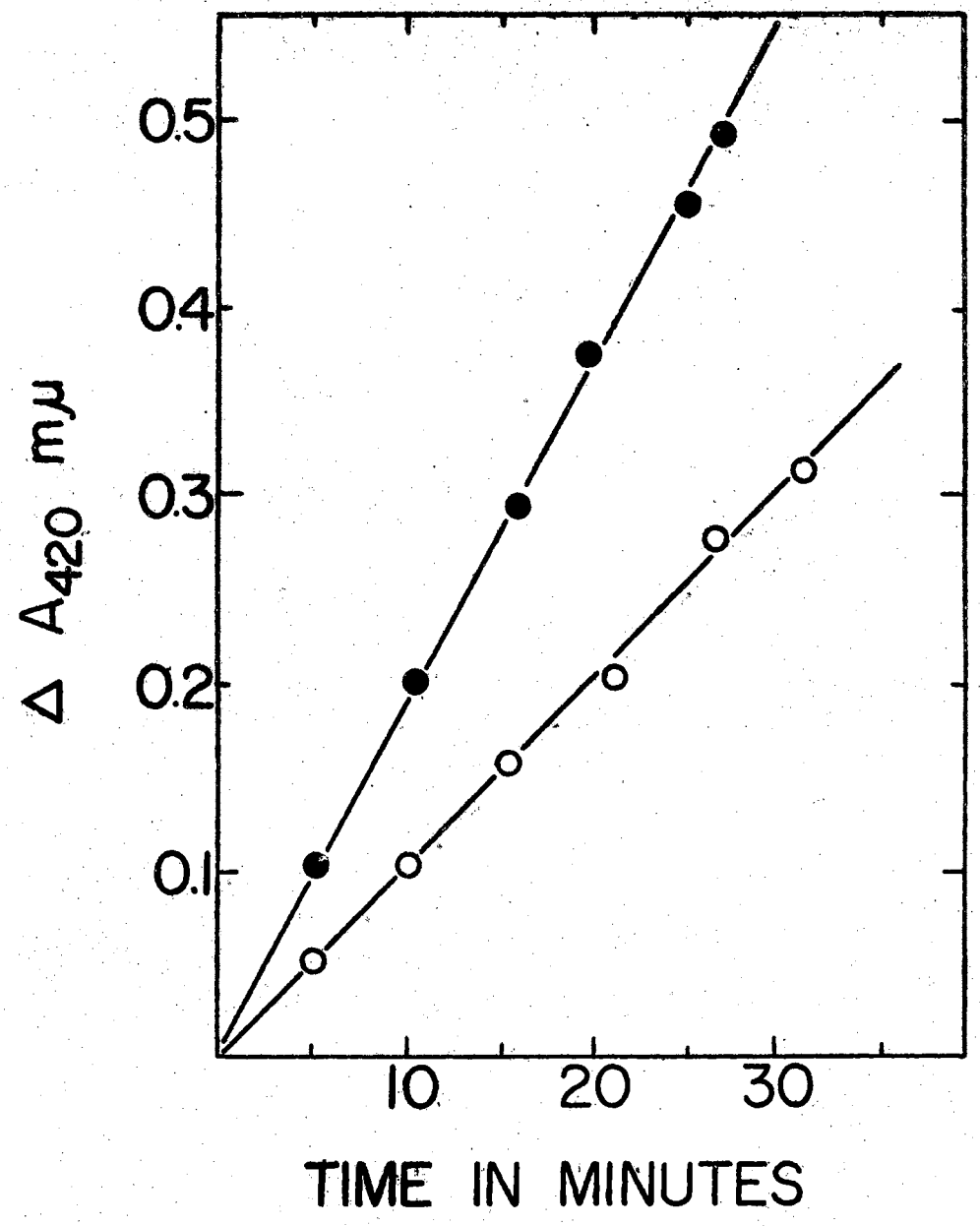

Figure 8. Spectrophotometric Determination of the Rate of Oxidation of Pyruvate by Liver Mitochondria.

O, 0.2 ml of a 1:5 dilution of mitochondrial suspension in 0.25 M sucrose. The 0.2 ml contained 0.093 mg N.

●, 0.4 ml of a 1:5 dilution of mitochondria suspension in 0.25 M sucrose. The 0.4 ml contained 0.2 mg N.

In 1963, Haslam and Krebs (90) showed that the rate of formation of $^{14}CO_2$ from pyruvate-1-^{14}C could be used to measure the amount of pyruvate-1-^{14}C converted to acetyl-CoA. This conclusion was valid only if a bicarbonate buffer was not used, and if the pyruvate concentration did not fall below 2 mM.

Pyruvate can also enter the tricarboxylic acid cycle via oxalacetate. Pyruvate-1-^{14}C entering the cycle by this route becomes oxalacetate-1,4-^{14}C. If this oxalacetate is then converted to citrate, carbons 1 and 4 of oxalacetate are converted to $^{14}CO_2$ when citrate is oxidized via the tricarboxylic acid cycle. Haslam and Krebs have calculated that the fixation of $^{14}CO_2$ into the tricarboxylic acid cycle intermediates cancels out the formation of $^{14}CO_2$ from the oxidation of isocitrate and α-ketoglutarate.

In experiments 4, 5 and 6 (Table VI) a vial of pyruvate-1-^{14}C was used which had been stored forzen for approximately a week. Therefore, the counts obtained as $^{14}CO_2$ are lower than those in experiments 1, 2, 3, 7, and 8, in which a freshly opened vial of pyruvate-1-^{14}C was used. Figures 9 and 10 show that the rate of $^{14}CO_2$ released was linear with time and proportional to the amount of mitochondria used.

An 8-fold range in phosphate concentration apparently had no affect upon the rate of decarboxylation of pyruvate-1-^{14}C. The ratio of activity of pyruvate dehydrogenase in "fed" and "fasted" mitochondria indicates that in fed animals the liver converts 20 to 25 percent more pyruvate to acetyl-CoA than do the livers of fasted animals. This figure is in agreement with the results obtained by the spectrophotometric assays.

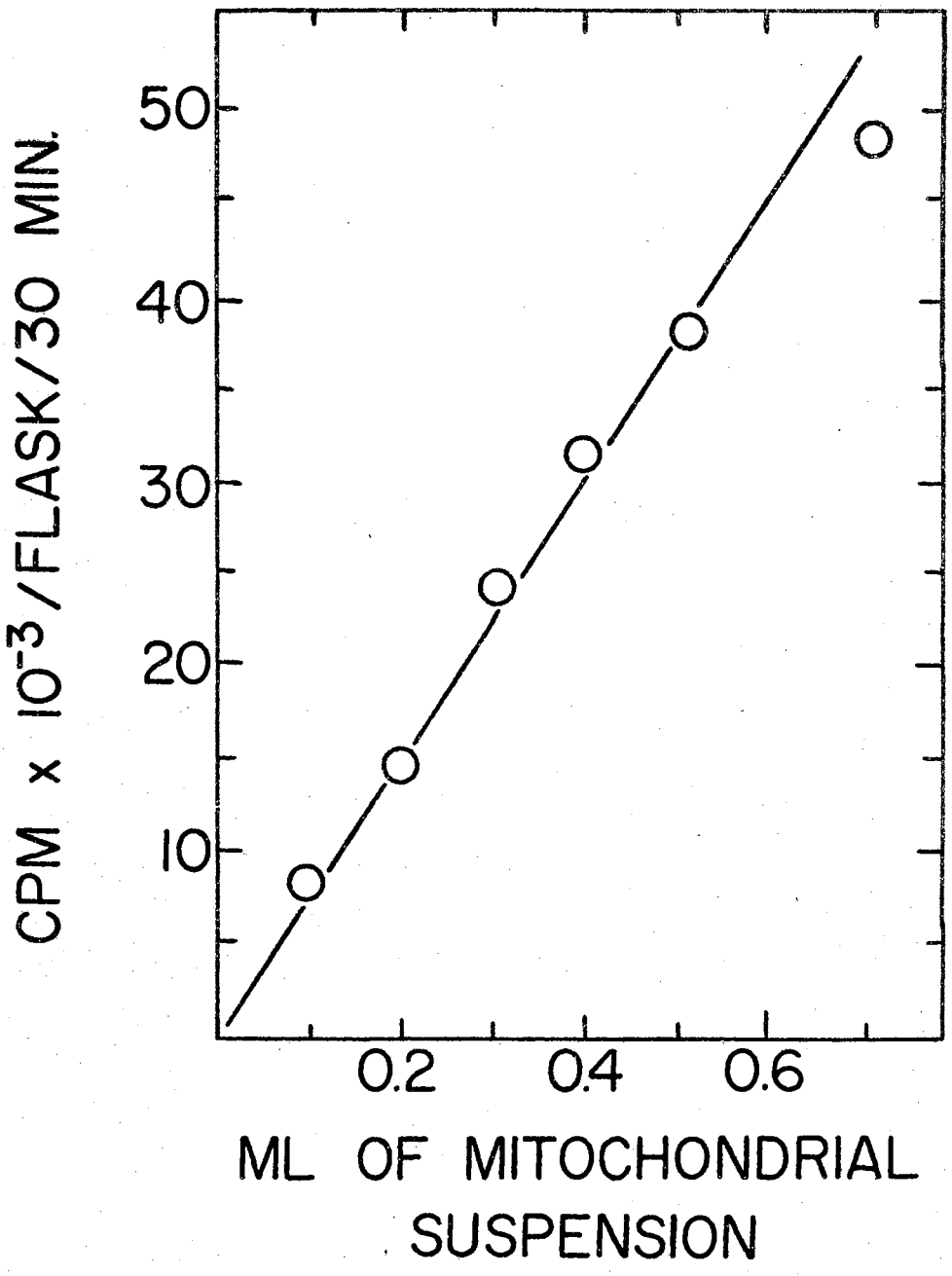

Figure 9. Rate of $^{14}CO_2$ Produced from Sodium Pyruvate-1-^{14}C by Rat Liver Mitochondria.

The assay mixture contained different concentrations of a 1:1 mitochondrial suspension in 0.25 M sucrose. All other additions were similar to those described in the text. 0.05 ml of suspension contained 0.15 mg N.

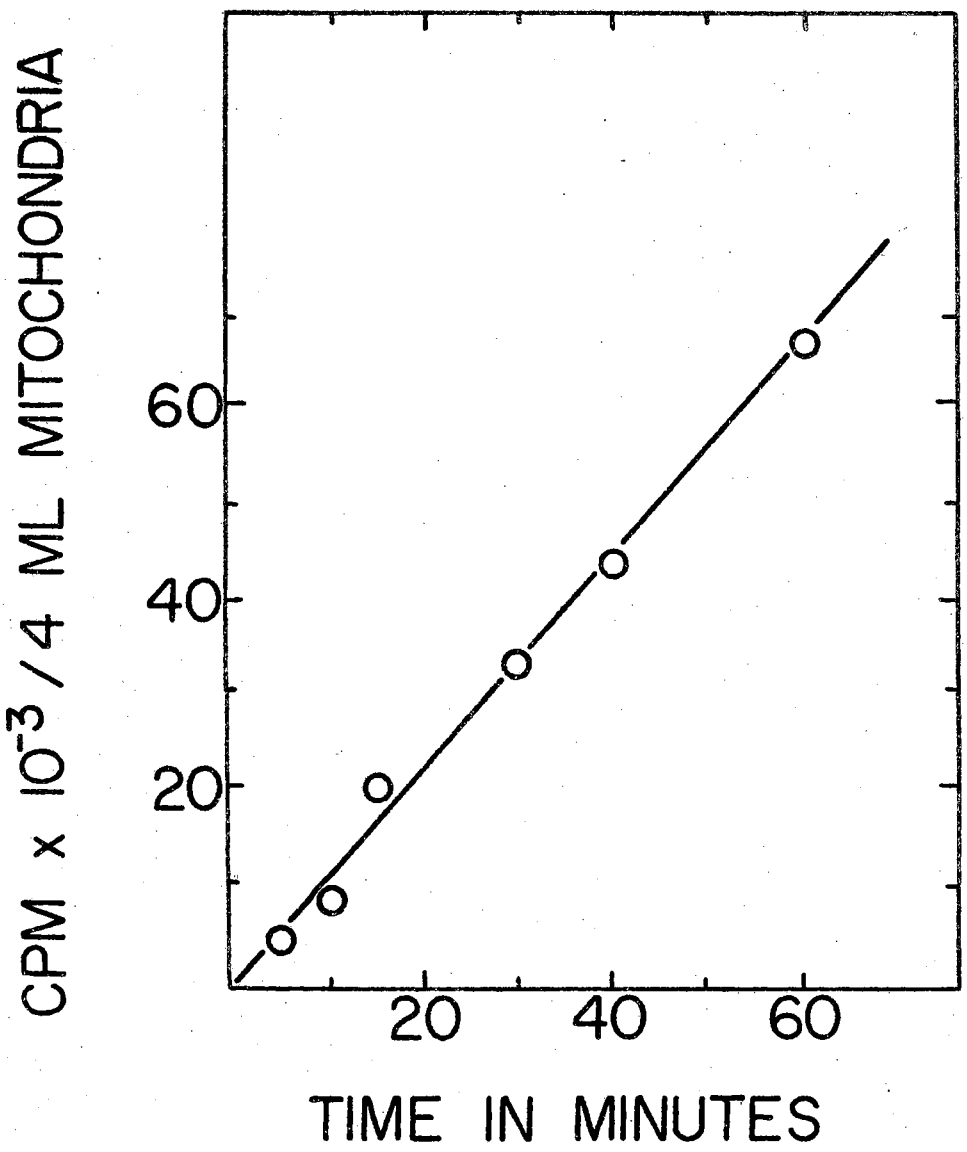

Figure 10. Rate of $^{14}CO_2$ Produced from Sodium Pyruvate-1-^{14}C by Rat Liver Mitochondria as a Function of Time.

The assay mixture contained 0.05 ml of a 1:1 mitochondrial suspension in 0.25 M sucrose. All other additions were similar to those described in the text. 0.05 ml of suspension contained 0.15 mg N.

Long Term Labeling Experiments in Vivo

The results of a series of pyruvate-2-^{14}C incorporation studies involving different mammalian species are shown in Table VII. All of the species studied show similar differences in glutamate labeling patterns as a result of feeding and fasting as do rats. Fasted animals produce glutamate with a very low percentage of total activity in carbon 5, and fed animals produce glutamate which has a much higher percentage of its total activity in carbon 5.

Glutamate from mice and hampsters had the same percentage labeling in carbon 5 as the glutamate from rats. Fed guinea pigs and rabbits, however, produced glutamate which had significantly more radioactivity in carbon 5 than did that obtained from fed rats; 59 to 67 percent vs 30 to 38 percent, respectively, for liver glutamate. Fasting decreased the percentage of total labeling in carbon 5 of glutamate to a lesser extent in rabbits and guinea pigs than in the other species studied.

The specific activities of liver glutamate were higher in the fasted animals than in the fed animals. Mouse 2 was given 1.6 times more radioactive pyruvate than mouse 1, but the specific activity of the liver glutamate from mouse 2 was 4 times greater than the specific activity of the glutamate from mouse 1. The specific activities of the liver glutamate isolated from the fasted hampsters and guinea pigs were 2 times greater than the specific activities of the liver glutamate from the corresponding fed animals. In all of these animals the weights of the livers, within a given species, were essentially equal irrespective of nutritional condition. The liver glutamate from the fasted rabbits had a specific activity only slightly greater than that of the fed

TABLE VII

LABELING PATTERNS IN CARBON 5 OF TISSUE GLUTAMATE AFTER THE ADMINISTRATION OF PYRUVATE-2-^{14}C TO DIFFERENT MAMMALIAN SPECIES

Animal Number	(hr) Time Fasted	Amount of Pyruvate-2-^{14}C Injected (μc)	(hr) Time of Exp.	% of total labeling in carbon 5 of glutamate			
				Carcass		Liver	
				Sp. Act. μc/mmole	% in C 5	Sp. Act. μc/mmole	% in C 5
R34[a,b]	0	35[d]	2	90	44	197	38
R35[a]	48	35	2	44.6	8	259	2
M1	0	12.5	2	38.4	46	116	39
M2	48	20.8	2	21.1	12	466	3
Rb1	0	45	2	9	5	19.1	56
Rb2	51	41	2	0.68	29	20.5	4
Rb3	61	50	2	1	38	34	11
Rb4	0	40	2	5.5	67	27.9	59
H1	0	23.5	2	63.6	54	242	33
H2	0	23.5	2	44	48	243	33
H3	48	29.4	2	35.9	14	510	5
G1	0	21.5	2	6.7	64	36.8	62
G2	48	20	2	3.4	24	81.2	7
R253	0[c]	35	2	23.1	32	136	13

a. Data from Koeppe et al. (28).

b. R refers to rat, M to mouse, Rb to rabbit, H to hampster, and G to guinea pig.

c. Rat 253 was fed a high fat diet for 5 days.

d. All animals were injected intraperitoneally except rabbits 1, 2, and 3 which were injected intraveniously.

rabbits. However, the weights of livers of the fed and fasted rabbits varied considerably (Table III, experimental section).

In all of the species studied, the muscle protein glutamate obtained from the fasted animals had a lower specific activity than did the glutamate of the corresponding fed animals.

The liver protein glutamate which was isolated from Rat 253 had 13 percent of its total activity in carbon 5. Rat 253 was fed a high fat diet for a period of five days.

A single dose of either hydrocortisone or 9-α-fluoroprednisolone to fed rats, prior to the injection of pyruvate-2-^{14}C, did not alter the labeling patterns of either liver or muscle glutamate from that which was obtained in normal fed animals (Table VIII).

Short Term Labeling Experiments in Vivo

The long term experiments in vivo are undesirable for measuring product-precursor relationships, because in 2 hours other precursors of acetyl-CoA such as fatty acids and amino acids could affect the labeling patterns obtained in glutamate. Also, differences in the rate of incorporation of glutamate into protein as well as differences in the total protein content of the liver could affect the specific activity of the glutamate isolated from the fed and fasted animals more than would differences in routes of pyruvate metabolism.

The liver glutamate labeling patterns obtained in the short term experiments are in good agreement with the values observed in the long term experiments (Table IX). As noted with the long term experiments, the specific activities of the liver glutamates are higher in the fasted

TABLE VIII

EFFECT OF GLUCOCORTICOIDS UPON THE LABELING PATTERNS IN GLUTAMATE AFTER THE ADMINISTRATION OF PYRUVATE-2-^{14}C TO FED RATS

Rat Number	μC Pyruvate-2-^{14}C Injected	Hormone Injected	Amount of Hormone Inj. mg/100 gm Body Weight	% of total labeling in carbon 5 of glutamate			
				Carcass		Liver	
				Sp. Act. μc/mmole	% in C_5	Sp. Act. μc/mmole	% in C_5
246[a]	20	Hydrocortisone	2.4	3.6	49	61	44
248[b]	21	Hydrocortisone	2.5	14.1	44	133	34
249[b]	22.5	9-α-fluoro-prednisolone	3	13.9	44	130	38
250[b]	22.5	9-α-fluoro-prednisolone	3	9.5	44	139	28

Total time of experiment was 2 hours.

a. Rats received hormone 24 hours prior to the injection of pyruvate-2-^{14}C.

b. Rats received hormone 4 hours prior to the injection of pyruvate-2-^{14}C.

TABLE IX

LABELING PATTERNS IN LIVER GLUTAMATE AND ASPARTATE SUBSEQUENT TO THE SHORT TERM METABOLISM OF PYRUVATE-2-^{14}C IN VIVO

Rat Number	(hr) Time Fasted	(μc) Amount of Pyruvate 2-^{14}C Injected	Time of Exp. min.	Distribution of ^{14}C in Glutamate and Aspartate						
				Glutamate				Aspartate		
				Total Act. mμc	Specific Activity μc/mmole	% of ^{14}C in C-5	C-1	Total Act. mμc	Specific Activity μc/mmole	% of ^{14}C in carboxyl carbons
265	0	10	6	87	5.95 (1.7)[a]	45	10	23.8	5.92 (0.5)[a]	39
263	0	25	12	117	7.8 (2.0)	37	15	20.4	4.54 (0.6)	46
266	0	10	18	24	1.5 (1.9)	32	15	18.2	4.0 (0.49)	55
277	48	10	6	150	8.34 (2.3)	0.96	3	34.5	4.94 (0.86)	17
279	48	10	10	56.2	3.6 (2.8)	2.0		11.2	2.13 (0.94)	13
264	48	25	12	278	15.3 (4.0)	9.0	10	56.6	10.0 (1.2)	29
278	48	10	18	45	3.1 (2.2)	2.0	5	19.2	2.9 (0.96)	17
304	144	20	12	38.5	3.43 (3.9)	49	12	29.6	4.64 (2.2)	43

a. μmoles of amino acid isolated per gm of liver.

animals than in the fed animals. In both the fed and fasted animals the total activity and the specific activity of glutamate decreased considerably as the time of the experiment increased from 6 to 18 minutes. The specific activities of aspartate isolated from the fed animals were only slightly higher than the values obtained from aspartate from the fasted animals. Rat 304, which was fasted for 6 days, lost 35 percent of its weight. Its carcass contained no epididymal fat. The glutamate labeling patterns were similar to the patterns obtained in fed rats.

The experiments described in Table X represent an approach toward measuring the effect, if any, of acetyl-CoA pool sizes on the labeling patterns obtained in glutamate. Pyruvate-1-^{14}C is incorporated into carbon 1 of glutamate only via oxalacetate. Because pyruvate-1-^{14}C is converted to unlabeled acetyl-CoA, glutamate is not labeled by pyruvate entering the tricarboxylic acid cycle by this route. Therefore, any acetyl-CoA from lipid or amino acid oxidation will not affect the labeling of glutamate by pyruvate-1-^{14}C. The rate of incorporation of pyruvate-1-^{14}C into liver glutamate is 2 to 3 times greater in the fasted animals than in the fed animals. All of the activity in glutamate is in carbon 1.

Butyrate-1-^{14}C was injected into several fed and fasted rats in order to measure the flux of acetyl-CoA, formed from lipid oxidation, through the tricarboxylic acid cycle. With the exception of Rat 296, there were essentially no differences in the specific activities of the glutamate isolated from the fed and fasted animals. The higher specific activity in the glutamate of Rat 296 may have been the result of a

TABLE X

LABELING PATTERNS IN LIVER GLUTAMATE AND ASPARTATE SUBSEQUENT TO THE SHORT TERM METABOLISM OF PYRUVATE-1-^{14}C OR BUTYRATE-1-^{14}C IN VIVO

Rat No.	Time Fasted (hr)	Isotope Injected	μC Inj.	Time of Exp. (min)	Distribution of ^{14}C in Glutamate and Aspartate							
					Glutamate					Aspartate		
					Total Act. (mμc)	Sp. Act. μc/mmole		% of ^{14}C C-5	C-1	Total Act. (mμc)	Sp. Act. μc/mmole	
298	0	Pyruvate-1-^{14}C	20	12	38	1.17	(2.8)[a]		99	9.86	1.8	(0.46)[a]
300	0	Pyruvate-1-^{14}C	20	12	38	1.48	(3.1)		100	12.1	1.75	(0.78)
299	48	Pyruvate-1-^{14}C	20	12	66	2.67	(3.0)		100	15.1	2.75	(0.59)
301	48	Pyruvate-1-^{14}C	20	12	49	3.57	(2.5)		100	13.5	2.76	(0.89)
296	0	Butyrate-1-^{14}C	25	12	656	18.6	(3.9)	79	17	134	13.4	(1.1)
302	0	Butyrate-1-^{14}C	25	12	363	11.3	(3.1)	71	28	59.7	12.2	(0.48)
287	48	Butyrate-1-^{14}C	25	12	140	6.0	(3.8)	77	23	13.3	7.14	[b]
303	48	Butyrate-1-^{14}C	25	12	211	9.3	(3.7)	71	28	111	19.4	(0.78)

a. μ moles of amino acid isolated per gm of liver.

b. Some aspartate was lost during the isolation procedure.

difference in route of injection of the labeled butyrate. If for some reason the labeled butyrate had been transported more rapidly to the liver in this rat than in the other rats, one would expect a greater incorporation of ^{14}C into glutamate. The difference also may have been the result of normal biological variation between animals.

The incorporation of pyruvate-2-^{14}C into acetoacetate in the liver was studied in Rats 285 and 286 (Table XI). Only the activity in carbon 3 of acetoacetate was measured. The amount of incorporation of pyruvate-2-^{14}C into the acetoacetate was very low, and essentially the same in the fed and fasted animals.

The incorporation of butyrate-1-^{14}C into acetoacetate and β-hydroxybutyrate was low compared to the amount of labeling incorporated into glutamate. The fasted animals incorporated slightly more labeling into the blood and liver ketone bodies than did the fed animals.

Pyruvate-2-^{14}C Incorporation Studies *In Vitro*

In general, the results obtained from the metabolism of pyruvate-2-^{14}C *in vitro* were qualitatively the same as those observed *in vivo* (Table XII). Homogenates and slices prepared from the livers of fed rats produced glutamate which had a higher percentage of its total labeling in carbon 5 than did the corresponding preparations from fasted rats.

Homogenates

Liver homogenates from a fed rat produced glutamate which had 75 to 76 percent of its total activity in carbon 5, and aspartate which

TABLE XI

INCORPORATION OF PYRUVATE-2-^{14}C AND BUTYRATE-1-^{14}C INTO CARBON 3 OF ACETOACETATE AND B-HYDROXYBUTYRATE

Rat No.	Isotope Injected	Amt. Inj. μc	Time of Exp. min.	Acetoacetate Liver mμC	Acetoacetate + β-hydroxybutyrate Liver mμc	Blood μc/3 ml
285	Pyruvate-2-^{14}C	21.25	12	0.72		
286	Pyruvate-2-^{14}C	21.25	12	0.83		
287	Butyrate-1-^{14}C	25	12	0.56		
296	Butyrate-1-^{14}C	25	12			0.65
295	Butyrate-1-^{14}C	25	12		5.57	1.35
297	Butyrate-1-^{14}C	25	12		2.14	

TABLE XII

INCORPORATION OF PYRUVATE-2-^{14}C INTO GLUTAMATE AND ASPARTATE BY RAT LIVER HOMOGENATES AND SLICES

Exp. No.	Time Fasted (hr)	Amt. of Pyruvate 2-^{14}C Inj. (μc)	Tissue Preparation	Time of Incubation (min)	Distribution of ^{14}C in Glutamate and Aspartate						
					Glutamate				Aspartate		
					μc/mmole Specific Activity		% of ^{14}C C-5	C-1	μc/mmole Specific Activity		% of ^{14}C in Carboxyl Carbons
2a	0	5	Homogenate [A]	15	46	(0.94)[H]	77	39	47	(0.2)	96
2b	0	5	Homogenate [A]	30	34	(1.2)	75	27	48	(0.8)	98
4a	48	5	Homogenate [A]	15			69				71
4b	48	5	Homogenate [A]	30			62				67
8a	48	2.5	Homogenate [B]	30			58				71
8b	48	2.5	Homogenate [B]	60			59	21			64
9a	48	2.5	Homogenate [B]	60	26	(1.93)	84	4			33
9b	48	2.5	Homogenate [B,C]	60	25	(1.93)	92	3			33
10	48	1.25	Slice [E]	30			59	3			6
11a	48	2.0	Slice [E]	6			58	2			8
11b	48	2.0	Slice [D,E]	6			86	2			6
12a	0	2.5	Slice [E]	6	109	(0.16)	77	3	69	(0.14)	12

TABLE XII (continued)

12b	0	2.5	Slice [E]	6	96	(0.22)	72	2	74	(0.16)	13
13	48	2.5	Slice [E]	6	105	(0.22)	59		131	(0.13)	9
14a	48	2.5	Slice [F]	1	27	(0.21)	38	1	85	(0.14)	5
14b	48	2.5	Slice [F]	6	86	(0.25)	53	3	83	(0.16)	9
15a	48	2.5	Slice [G]	0.3	14	(0.23)	42	3	30	(0.16)	8
15b	48	2.5	Slice [G]	6	95	(0.21)	38	5	146	(0.27)	17
16a	0	2.5	Slice [G]	0.8	12.5	(0.41)	83	3	13	(0.19)	19
16b	0	2.5	Slice [G]	6	79	(0.41)	74	7	74	(0.34)	45

A. Incubation medium contained 40 μmoles K-pyruvate.
B. Incubation medium contained 6.3 mg cytochrome C.
C. Incubation medium contained 40 μ moles octonoate.
D. Incubation medium contained 40 μ moles fumarate.
E. Incubation medium was KRB buffer.
F. Incubation medium was KRP buffer.
G. Incubation medium was KRP-Ca++ buffer.
H. Total mg of amino acid obtained from liver slices or homogenates.

had 96 to 98 percent of its total activity in carbons 1 and 4. The glutamate obtained from the liver homogenate of a rat which had been fasted 48 hours contained 62 to 69 percent of its total activity in carbon 5. Carbons 1 and 4 aspartate contained 67 to 71 percent of the total activity present in this amino acid. Adding cytochrome C to the homogenates obtained from fasted rats lowered the percentage incorporation of pyruvate-2-^{14}C into carbon 5 of glutamate to 59 percent. However, the addition of cytochrome C to the incubation medium did not affect the labeling patterns in aspartate.

The length of the incubation did not appreciably affect the labeling patterns obtained in either glutamate or aspartate. However, preincubation at 37^{o} for 10 minutes prior to the addition of pyruvate-2-^{14}C markedly raised the percentage incorporation of pyruvate-2-^{14}C into carbon 5 of glutamate. Preincubation of the homogenate with octanoate increased the percentage total labeling in carbon 5 to 92 percent. This value is to be compared with experiment 9a in which the glutamate had 84 percent of its total activity in carbon 5 and to experiment 8a in which the glutamate had 59 percent of its labeling in carbon 5.

Slices.

Incubation of "fasted" liver slices for 6 to 30 minutes in either KRP or KRB buffer did not markedly affect the labeling patterns of glutamate or aspartate. In general, "fasted" slices produced glutamate which contained 53 to 59 percent of its total labeling in carbon 5 and aspartate which had 5 to 9 percent of its total activity in carbons

1 and 4. "Fed" slices produced glutamate which had 72 to 78 percent of its total activity in carbon 5 and aspartate which had 12 to 13 percent of its total activity in carbons 1 and 4. Liver slices incubated with the above buffers incorporated less activity into the carboxyl's of aspartate than did liver homogenates. Also, liver homogenates incorporated more activity into carbon 1 of glutamate than did liver slices.

Addition of fumarate to the incubation medium of the liver slices caused the percentage labeling into carbon 5 of glutamate to increase from 59 to 86 percent.

Incubation of "fasted" liver slices in KRP buffer for a shorter time period, i.e., 1 minute vs 6 minutes, resulted in the production of glutamate which had a lower percentage of activity in carbon 5 of glutamate; 38 vs 53 percent, respectively (see experiment 14a and 14b).

"Fasted" slices incubated in KRP minus Ca++ produced glutamate which had only 35 percent of its activity in carbon 5, and aspartate which had 17 to 19 percent of its activity in carbons 1 and 4. "Fed" slices incubated in the same medium yielded glutamate which had 83 percent of its activity in carbon 5, and aspartate which had 45 percent of its activity in carbons 1 and 4. Incubations of "fed" and "fasted" liver slices for less than one minute lowered the percentage labeling in carbons 1 and 4 of aspartate, but did not affect the labeling patterns in glutamate.

CHAPTER IV

DISCUSSION

Long Term Experiments *In Vivo*

It has been shown repeatedly by Koeppe *et al.* (28) and Freedman *et al.* (30) that the labeling patterns obtained in glutamate, following the administration of pyruvate-2-^{14}C, differ markedly in fed and fasted rats. In the fed animals 30 to 40 percent of the total labeling in liver glutamate is in carbon 5. Rats fasted for 24 to 48 hours synthesize liver glutamate which has only 2 to 4 percent of its total activity in carbon 5. It has been postulated that the labeling patterns in glutamate are indicative of the relative amounts of pyruvate converted to acetyl-CoA and to oxalacetate (27). Our studies with different mammalian species show that changes in the labeling patterns of glutamate as a function of the nutritional state of the animal are not unique to rats (Table VII), Benevenga *et al.* (91) have shown that dairy calves also yield glutamate whose labeling patterns are dependent upon the dietary condition of the animal. Fed rabbits and guinea pigs incorporate a larger amount of labeling from pyruvate-2-^{14}C into carbon 5 of glutamate than do rats, mice, or hampsters (Table VII). Assuming no great changes in the "pool"* sizes of acetyl-CoA and oxalacetate,

*The "pool" size of a specific compound refers not only to the concentration of the compound within the cell, but also to the net flux of precursor compounds through the pool.

the data indicate that with respect to the amount of pyruvate converted to oxalacetate, rabbits and guinea pigs convert more pyruvate to acetyl-CoA which is available to the tricarboxylic acid cycle than do the other species studied.

Evidence supporting the thesis that the labeling patterns in the liver glutamate of guinea pigs are a reflection of the amount of acetyl-CoA from pyruvate being made available to the tricarboxylic acid cycle is provided by the data of Hardwick and Lowenstein (92). They have shown that the rate of fatty acid synthesis in the guinea pig is one-tenth of the rate observed in rats. This decreased rate of fatty acid synthesis from acetyl-CoA is a result of the low activity of citrate cleavage enzyme in the guinea pig. If less citrate is being transported out of the mitochondria to provide acetyl units for fatty acid synthesis, more acetyl-CoA from pyruvate will be available for incorporation into glutamate. That is, if a citrate molecule is transported out of the mitochondria and cleaved to acetyl-CoA and oxalacetate, the acetyl-CoA is incorporated into fatty acid, whereas, the oxalacetate is transported back into the mitochondria, donating its activity to the oxalacetate "pool." On the other hand, the acetyl-CoA "pool" as a result of the loss of one acetyl-CoA molecule is decreased in total activity.

The specific activity of the glutamate isolated from the liver protein of the fasted mammals is 2 to 4 fold greater than that of the glutamate isolated from the livers of the fed mammals. The fact that more pyruvate is incorporated into the liver glutamate of the fasted animals provides tenative evidence for the postulation that glutamate labeling patterns are a reflection of the relative routes of pyruvate

metabolism in the tricarboxylic acid cycle. However, since these were long term experiments, and since the glutamate was isolated from liver protein, the specific activities of the glutamates may also be dependent upon the relative rates of incorporation of the amino acids into liver protein.

The glutamate isolated from the muscle protein of the fasted animals had a lower specific activity than did the muscle glutamate from the fed animals (Table VII). This may be the result of less pyruvate-2-^{14}C being available to the muscle of the fasted animals because more pyruvate is being used by the liver, or it may be that less glutamate is being incorporated into the muscle protein of the fasted animals.

It was of interest to determine if the gluconeogenic hormones could affect the metabolism of pyruvate. Fed rats were administered either hydrocortisone or 9-α-fluoroprednisolone. Four to 12 hours after the administration of the hormones the animals were injected with pyruvate-2-^{14}C. Under the conditions of this experiment neither hormone affected a change in the labeling patterns of glutamate.

Rat 253 which had been fed a high fat diet for 5 days produced glutamate which had only 13 percent of its total labeling in carbon 5 of glutamate (Table VII). The animal had a liver glycogen content which was comparable to that found in normal fed rats (Table II).

From the long term experiments it can be concluded that the changes in labeling patterns in glutamate as a function of the diet are a common phenomenon in all mammals. Under the conditions of our experiments the gluconeogenic hormones hydrocortisone and 9-α-fluoroprednisolone do not affect significant changes in glutamate labeling patterns. Lardy et al.

(93) observed an increase in the activity of P-enolpyruvate carboxykinase in an adrenalectomized rat 4 hours after the injection of a single dose of hydrocortisone, but it took 5 days of multiple doses of hydrocortisone to affect a maximum increase in the activity of the enzyme (43). A comparable increase in the activity of the enzyme was obtained after 48 hours of fasting. The fact that the gluconeogenic hormones did not alter the labeling patterns in glutamate, coupled with the observations of Hahn (83) that only 24 hours of fasting were required to change the labeling patterns in glutamate, indicate that factors other than the activity of P-enolpyruvate carboxykinase are responsible for the changes in the labeling patterns of glutamate. The data obtained with the animal fed a high fat diet suggest that the labeling patterns in glutamate are related to the extent to which animals are oxidizing fatty acids.

Pyruvate Dehydrogenase

The activity of pyruvate dehydrogenase was measured in the liver mitochondria of fed and fasted rats to determine if this enzyme has any function in the control of pyruvate metabolism (Tables V and VI). It was found that the activity of the enzyme in fed rats was about 25 percent greater than in fasted rats. This decrease in activity of pyruvate dehydrogenase in fasted rats supports the thesis that the decreased percentage of labeling in carbon 5 of glutamate is, in part, a result of less pyruvate entering the cycle via acetyl-CoA. However, this change in enzymatic activity may be due to inhibition by acetyl-CoA or fatty acids rather than changes in enzyme concentrations.

Garland and Randle (56) have shown that acetyl-CoA inhibits pig heart pyruvate dehydrogenase. Recently, Walter, Paetkau, and Lardy (49) have shown that caprylate markedly inhibits the decarboxylation of pyruvate, and they suggest that because of this inhibition pyruvate is preferentially carboxylated to oxalacetate.

Pyruvate-2-^{14}C Incorporation Studies *In Vitro*

The incorporation of pyruvate-2-^{14}C into glutamate by liver slices and homogenates was investigated to determine if these were satisfactory systems for studying the effects of various conditions upon the labeling patterns of glutamate (Table XII). In all of the systems studied the percentage of total labeling in carbon 5 of glutamate was higher than the values obtained *in vivo*. It is known that the rate of carbohydrate synthesis in liver slices or homogenates is very low and only a fraction of the rate *in vivo* (3).

The aspartate isolated from the homogenates had a large percentage of its total activity in carbons 1 and 4. The large percentage of labeling in carbon 5 of glutamate and carbons 1 and 4 of aspartate produced by the homogenates indicates that the mechanisms controlling pyruvate metabolism are destroyed when the integrity of the cell is disrupted by homogenization.

The aspartate obtained from the liver slices which were incubated in KRP or KRB buffer had only a small percentage of its total activity in carbons 1 and 4. However, the slices that were incubated in KRP minus Ca^{++} produced aspartate which had labeling patterns similar to the ones found *in vivo*. The incubation medium used in the homogenate

studies contained no Ca^{++}. It is a well known fact that Ca^{++} inhibits oxidative phosphorylation in isolated mitochondria (94). Ca^{++} also inhibits pyruvate kinase (95) and, according to Gevers and Krebs (51), pyruvate carboxylase. Our data indicate that Ca^{++} also inhibits the metabolism of pyruvate in liver slices. The labeling patterns in aspartate suggest that Ca^{++} is preventing the tricarboxylic acid cycle from cycling properly.

The very high percentage of labeling in carbon 5 of the glutamate formed in the slices incubated with fumarate, and the fact that aspartate is accumulating during the incubation suggest that the carboxylation of pyruvate is being inhibited by feedback inhibition.

The labeling patterns produced in liver glutamate and aspartate *in vitro* change as a result of the dietary condition of the animal. However, they are greatly influenced by added substrate, preincubation, and the type of buffer used.

Short Term Experiments *In Vivo*

The labeling patterns obtained in liver glutamate and aspartate as a result of the short term metabolism of pyruvate-2-^{14}C *in vivo* are consistent with the results obtained in the long term experiments (Table IX). The liver glutamate obtained from the fasted rats had twice the specific activity of the glutamate from fed rats. These data are consistent with the results obtained in the long term experiments, and indicate that, in comparison to fed rats, fasted rats incorporate more ^{14}C into liver glutamate. The results obtained with pyruvate-1-^{14}C also indicate that relatively more pyruvate is converted

into oxalacetate in the fasted animal than in the fed (Table X).

It may be argued that the increase in specific activity in glutamate as well as the changes in labeling patterns in glutamate in the fasted rat are a result of fluctuations in the size of the oxalacetate "pool." That is, if one assumes that in the livers of fed and fasted rats the same amount of pyruvate is oxidized to acetyl-CoA, then the specific activity of the oxalacetate which is incorporated into glutamate would affect the percentage labeling of the carbons of glutamate. If oxalacetate of high specific activity is incorporated into glutamate, carbon 5 of glutamate will contain a lower percentage of the total activity. On the other hand, if the oxalacetate had a low specific activity, the relative amount of labeling in carbon 5 of glutamate would be higher. The results in Table IX show that the percentage of total labeling in carbon 5 of glutamate from a fed rat is 35 to 40 percent. In a fasted rat this value is decreased to 2 percent. The effective "pool" size of liver oxalacetate in the fed rat would have to be at least 10 fold that in the fasted rat for the observed changes in percentage labeling in carbon 5 of glutamate to be due to changes in the oxalacetate "pool." Correspondingly, the specific activity of glutamate and aspartate would have to be considerably more in the fasted rat than in the fed rat. From the data in Tables IX and X it can be seen that the specific activity of glutamate in fasted rats given pyruvate-2-^{14}C or pyruvate-1-^{14}C is only 2 to 3 fold that of the glutamate from fed rats. The specific activities of the aspartates from fed and fasted rats injected with pyruvate-2-^{14}C are essentially the same. The aspartate from the fasted rats injected with pyruvate-1-^{14}C is only 2 to 3

fold that of the aspartate from the fed rats. These data support the thesis that changes in the "pool" size of oxalacetate are not responsible for the changes in the labeling patterns of glutamate. Further evidence supporting this conclusion is the fact that the liver oxalacetate levels do not change in 24 hours of fasting or in light diabetes (58,60,61). The increased specific activity of glutamate in fasted rats is consistent with the observation that in fasted and diabetic animals there is an elevated incorporation of pyruvate and CO_2 into blood glucose via oxalacetate (67,68,96,97,98).

The thesis that the increased incorporation of pyruvate into oxalacetate is followed by a decrease in the conversion of pyruvate to acetyl-CoA is supported by the following discussion. As mentioned previously pyruvate dehydrogenase is markedly inhibited by short chain fatty acids and acetyl-CoA (49,56), and pyruvate dehydrogenase activity in "fasted" mitochondria is less than in "fed" mitochondria (Tables V and VI). Renold et al. (68) have shown that, although the overall disappearance of pyruvate is the same in slices from fed and fasted livers, 1.6 times more pyruvate is converted into glucose by the "fasted" slices than by the liver slices from the fed rats. It has also been observed in liver slices incubated with carnitine, which is known to accelerate fatty acid oxidation, that the incorporation of alanine into glucose and glycogen is increased with no effect on the total alanine uptake (99). Krebs, Speake, and Hems (100) have shown that short chain fatty acids accelerate glucose formation from lactate in kidney slices.

In recapitulation, it has been shown that, with respect to the rates observed in normal fed animals, dietary conditions such as dia-

betes and starvation cause an increase in the rate of carboxylation of pyruvate to oxalacetate and a decrease in the rate of oxidation of pyruvate to acetyl-CoA. It has been shown that the labeling patterns in glutamate fluctuate in accordance with the observed changes in the routes of pyruvate metabolism through the tricarboxylic acid cycle, and not as a result of changes in the cellular concentration of oxalacetate.

Even though evidence has been presented which shows that in fasted animals less pyruvate is oxidized to acetyl-CoA, the possibility exists that the labeling patterns in glutamate are affected by changes in the effective size of the acetyl-CoA "pool." Wieland et al. (58) did not observe any increase in the concentration of acetyl-CoA in the livers of fasted rats; however, a net increase in the flux through acetyl-CoA would effectively be the same as increasing the pool size of acetyl-CoA. The acetyl-CoA-1-^{14}C from pyruvate-2-^{14}C would be diluted out by "cold" acetyl-CoA resulting from the oxidation of fatty acids, if there were a greater net flux of "cold" acetyl-CoA through the tricarboxylic acid cycle, and the relative percentage of labeling in carbon 5 of glutamate would of necessity be lowered.

In order to determine if fluctuations in the "pool" size of acetyl-CoA or in the net flux of acetyl-CoA through the tricarboxylic acid cycle affect the labeling patterns in glutamate, butyrate-1-^{14}C was injected into fed and fasted rats (Table X). It was found that the incorporation of butyrate-1-^{14}C into the liver glutamate was essentially the same in fed and fasted rats. From these data it can be concluded that the decreased labeling in carbon 5 of glutamate in fasted rats is

not due to dilution by "cold" acetyl-CoA which is produced by the oxidation of fatty acids, and that the labeling patterns in glutamate are a reflection of the relative routes of pyruvate metabolism through the tricarboxylic acid cycle. The results obtained with the butyrate injected animals is in agreement with the data of several investigators who have shown that the rate of oxidation of short chain fatty acids is essentially the same in fed and fasted rats (16). It has been shown that short chain fatty acids are oxidized more rapidly than is palmitate (16). Therefore, the rate of oxidation of butyrate-1-^{14}C should be indicative of the maximum flux of acetyl-CoA through the tricarboxylic acid cycle.

The fact that the total radioactivity of the ketone bodies isolated from the livers of fed and fasted rats given pyruvate-2-^{14}C or butyrate-1-^{14}C is very low in comparison to the amount of ^{14}C incorporated into glutamate indicates that relatively small amounts of these compounds are being incorporated into ketone bodies via the condensation of two acetyl-CoA molecules.

It has been postulated that fatty acids are the main source of energy for gluconeogenesis and that they play a decisive role in "switching on" gluconeogenesis by inhibiting pyruvate oxidation, by stimulating pyruvate carboxylation, and by promoting malate formation (49). The close relationships between glutamate labeling patterns and the extent of lipid oxidation in the animal is shown by the labeling patterns obtained in Rat 254, which was fed a high fat diet for 5 days, and rat 304, which had been fasted 6 days prior to the administration of pyruvate-2-^{14}C (Tables VII and IX, respectively). Feeding a diet high in fat

resulted in a lowering of the percentage labeling in carbon 5 of glutamate to 13 percent. The labeling patterns in the liver glutamate of the 6 day fasted rat were the same as those found in fed rats. The long term fasted rat contained no epididymal fat, and had decreased 39 percent in body weight. Chernick and Scow (101) could measure no body fat in 8 day fasted pancreatectomized rats which had decreased 32 to 36 percent in body weight. They stated that several of their animals died because the rate of gluconeogenesis, as a result of markedly depleted protein stores, was insufficient to maintain adequate blood glucose levels. Further evidence supporting the hypothesis that the labeling patterns in glutamate are closely associated with gluconeogenesis and lipid oxidation is provided by the data of Hahn (83). She has shown that 2 gm of glucose given by stomach tube to a fasted rat 30 minutes before the injection of pyruvate-2-^{14}C increased the percentage labeling in carbon 5 of liver glutamate to 24 percent. Koeppe et al. (31) found that glucose administration by stomach tube to fasted rats 30 minutes prior to isotope injection did not consistently result in "fed" labeling patterns in muscle glutamate from lactate-2-^{14}C. Lossow and Chaikoff (102) and McCalla et al. (103) have shown that the administration of glucose to starved rats resulted in a reduction of palmitate conversion to CO_2 to a value which was about one-eighth that observed in the fasted rat. The results of Koeppe et al. (31) can be explained by the data of Lewis, Allen, and Weinhouse (104). The emphasized the rapidity with which lipid oxidation can be suppressed by glucose administration, and indicated that, unless relatively large amounts of glucose are given, the enhanced rates of lipid oxidation are quickly re-established.

CHAPTER V

SUMMARY

The labeling distribution in muscle and liver protein glutamate and aspartate 2 hours after the intraperitoneal injection of pyruvate-2-^{14}C has been studied in fed and fasted rabbits, hampsters, mice, and guinea pigs. The labeling patterns in these amino acids are consistent with the data obtained previously in fed and fasted rats. The percentage of total labeling in carbon 5 of glutamate and carbons 1 and 4 of aspartate is low in the fasted mammals and high in the fed mammals.

It has been found that neither hydrocortisone nor 9-α-fluoroprednisolone when injected into fed rats 4 to 12 hours prior to the administration of pyruvate-2-^{14}C affected the labeling patterns in liver and muscle protein glutamate and aspartate. Feeding a high fat diet lowered the percentage of total labeling in carbon 5 of liver protein glutamate to 13 percent.

The oxidative decarboxylation of pyruvate was studied *in vitro* in liver mitochondria isolated from fed and fasted rats with the following three assays: 1. A spectrophotometric method in which ferricyanide was used as the electron acceptor. 2. Measurement of O_2 uptake in a Warburg apparatus. 3. Measurement of $^{14}CO_2$ production from pyruvate-1-^{14}C. It was found that less pyruvate is decarboxylated by liver mitochondria of fasted rats than of fed rats.

The short term (6 to 18 minutes) incorporation of pyruvate-2-^{14}C *in vivo* into the free glutamate and aspartate of rat liver was investigated. In all of the short term experiments the radioactive compounds were injected intraperitoneally. The labeling patterns obtained in the free glutamate and aspartate were consistent with the patterns obtained in the long term experiments. The glutamate from the livers of the fasted rats had a higher specific activity than the glutamate from the fed rats.

Short term labeling experiments were conducted using pyruvate-1-^{14}C and butyrate-1-^{14}C. Fasted rats administered pyruvate-1-^{14}C produced glutamate whose specific activity was 2 to 3 fold greater than the specific activity of the glutamate from fed rats. The specific activities of the glutamates obtained from fed and fasted rats injected with butyrate-1-^{14}C were essentially equal.

The incorporation of pyruvate-2-^{14}C *in vitro* into glutamate and aspartate was studied using liver slices and homogenates from fed and fasted rats. These investigations showed that the labeling patterns *in vitro* in glutamate and aspartate have the same qualitative relationships as obtained in these amino acids *in vivo*. The labeling patterns fluctuated with the nutritional status of the animal, but the percentage of total labeling in carbon 5 of glutamate was considerably higher in the experiments *in vitro*.

The data from the above experiments indicate that the changes in glutamate labeling patterns as a result of feeding and fasting is a phenomenon which is probably common to all mammalian species and that the labeling patterns in glutamate following the administration of

pyruvate-2-^{14}C are indicative of the relative amounts of pyruvate being converted to acetyl-CoA and oxalacetate.

A short term experiment in vivo has been described which may be useful in assessing the effects of hormones, antimetabolites, different nutritional conditions, etc. upon the rates of gluconeogenesis and glycolysis in the liver of intact animals. The experiments can be performed rapidly and yield results which are dependent upon the integrated control mechanisms of the body.

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